Rice response to simultaneous biotic and abiotic stresses

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Abstract

With the predicted climate change and an ever-growing population there is increasing pressure to develop crop plants with improved stress responses, increased yield and high nutritive value. We have explored transcriptomic changes in the leaves and roots of rice plants (*Oryza sativa* japonica cv Nipponbare) in response to drought and the root-knot nematode *Meloidogyne graminicola*. A glasshouse model was developed to mimic conditions experienced by rice plants in the field. The plant responses under simultaneous biotic and abiotic stress were dominated by the drought element accompanied by a unique set of genes that were only responsive to the simultaneous stress. Highlighted within this group were novel members of stress-responsive gene families for example cytochrome P450, wall-associated kinases, lipid transfer protein-like proteins and new candidate genes that may play important roles in the response of rice to multiple stresses. The genes that were differentially regulated between the multiple and the drought stress treatment were explored using loss-of-function mutants. The loss-of-function mutant for peroxidase precursor gene (*per*) showed improved growth and yield compared to the wildtype Nipponbare plants. The experiments conducted in growth rooms were validated in a field study. Both Nipponbare rice plants, and the popular lowland indica rice cv IR64 were grown under prolonged vegetative drought stress accompanied by cyst nematode or root-knot nematode infection.

Reduction of phytate, an anti-nutrient, has been adopted as a major strategy to improve the nutritional value of crop plants. Nematode susceptibility of low phytate Arabidopsis plants was studied to determine the effect of reduced phytate content on the plant's defence response.

The study has provided insight into the genome-wide transcriptional changes in rice under a combined biotic and abiotic stress. It has led to better understanding of the stress responses in plants that will be advantageous in developing crop varieties with improved yield and nutritive value.

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1 Chapter

Introduction

Being sessile, plants need to continuously respond and adapt to the changing environment for survival. Plants experience a wide range of environmental perturbances during development that could limit their productivity. When plants are grown under sub-optimal environmental conditions, a yield gap is observed and thus the actual average yield obtained is much lower than the maximum yield potential of the particular crop (Lobell, Cassman and Field 2009). The yield gaps for three major cereal crops; wheat, rice and maize are 40 %, 75 % and 30 % respectively, in major growing areas of the world (Fischer, Byerlee and Edmeades 2009). The major factors responsible for the yield gap in crop species can be classed as: (i) abiotic factors, such as excessive or insufficient temperature, water or minerals or (ii) biotic factors, such as bacterial, fungal or insect attack (Gaspar *et al.* 2002). These environmental stresses are responsible for large-scale crop loss each year and with the predicted climate change, such losses are expected to increase. Nearly 50 % of crop yield losses each year are contributed by the abiotic stresses (Wang, Vinocur and Altman 2003). The predicted climate change, characterised by an increase in temperature, increased concentration of green-house gases, intensified hydrologic cycle and increase in tropospheric ozone levels will have a multifaceted effect on crop growth and productivity. The results from Free Air Carbon dioxide Experiments (FACE) have established that increase in CO_2 levels in the atmosphere will lead to photosynthetic carbon gain, increased nitrogen use efficiency, decreased water use in the leaves, but the yield gain in crop species will be much smaller than anticipated (Leakey *et al.* 2009). Also the change in hydrological cycle will cause frequent extreme events of floods and storms in coastal areas accompanied by drought and reduced soil moisture in the drier regions, resulting in reduced productivity (Schmidhuber and Tubiello 2007). The anticipated rise in temperature will lead to a shorter life cycle and increased biomass in plants. Temperature changes outside the typical range during the major growth stages of crop plants will highly affect productivity (Moriondo, Giannakopoulos and Bindi 2011). Currently, pest and pathogens account for 15 % of the annual crop loss across the globe (Maxmen 2013). The increase in temperature and precipitation will alter the geographic distribution and

host range of various pests and pathogens (Newton, Johnson and Gregory 2011). The predicted changes will leave crop plants vulnerable to a large number of biotic and abiotic environmental stresses, acting upon them simultaneously. The world population is expected to increase by another 2 billion by 2050, and to assure food security for nearly 9 billion humans, agricultural produce will have to be increased by nearly 60 % of the 2006-2007 produce values. On the contrary, cereal production has barely increased by 2.3 % since 2006 (FAO 2013) and the increase in food grain production is not sufficient to attain food security for the growing world population. Apart from increased food grain production, availability of sufficient, safe and nutritious food that meets dietary needs and food preferences of individuals is a vital dimension of global food security (Panagariya 2002). By 2015, 560 million people in developing countries will remain under-nourished, lacking 100–400 kcal per day. This energy malnutrition is intensified by deficiency of macro-nutrients and micro-nutrients required for proper growth and development of an individual. Nearly 1.5 million people, especially women and children, suffer from iron deficiency, 740 million suffer from iodine deficiency and another 2.8 million children suffer from vitamin A-related blindness with a total of 200 million affected individuals across the globe (FAO 2000). Reduced concentration and bioavailability of these nutrients in the monotonous cereal consumption is a major cause of the deficiencies (Bhutta, Salam and Das 2013). Thus, to fulfil the food requirement of the world's growing population, it is important to understand the responses of crop plants to the simultaneous biotic and abiotic stresses and to implicate the acquired knowledge for improvement of crop yields and nutrition for attaining food security.

1.1. Rice

Rice (*Oryza* spp) is an important cereal crop which is the staple diet of about half of the world population. This figure is set to increase since the prediction suggests that demand for rice will increase by 2,000 million metric tonnes by 2030 (FAO). 90 % of rice is grown and consumed in the Asia-pacific region, cultivated in flooded lowlands through to rain fed dry land (Alexander Sarris 2004; Pandey 2009).

The genus *Oryza* originated 130 million years ago in the super continent Gondwanaland and the domestication of wild rice varieties started 9000 years ago, independently and concurrently at various sites in Asia and Africa. There are two cultivated species of rice with 21 wild rice species. The cultivated rice species, *Oryza sativa* and *Oryza*

glaberrima present a classic example of parallel evolution of a crop plant, sharing the same common ancestor but following two different evolutionary paths. *Oryza sativa*, also known as the Asian rice, is grown worldwide whilst *O. glaberrima*, the African rice, is grown in only certain parts of West Africa. The *O. sativa* cultivars are further divided into two varietal groups or sub species, japonica and indica. There are mixed opinions on the origin of these two varietal groups. Phylogeographic analysis of the DNA sequences from these varieties indicates that they may be the result of two independent domestication events of the same ancestor *O. rufipogon. O. sativa* indica was domesticated in eastern India, Myanmar and Thailand, whereas *O. sativa* japonica was domesticated in southern China (Londo *et al.* 2006). The most recent modelling, however, based on SNP data suggests that the two varietal groups originated from a single domestication event that took place ~8,200 to 13,500 years ago in the Yangtze valley in China (Molina *et al.* 2011). The International Rice Research Institute (IRRI) has classified rice ecosystems into four main types; irrigated rice ecosystem, rainfed lowland rice ecosystem, upland rice ecosystem and the flood-prone ecosystem (IRRI, 1993). Rice cultivation and productivity is water dependent and thus highly susceptible to drought and floods. The major rice producing belts in Asia produce only 40 % of total production efficiency due to damage caused by drought (Alexander Sarris 2004; Farooq *et al.* 2009). Over 75 % of the world's rice growing area is irrigated rice ecosystem (Halwart and Gupta 2004). The rainfed rice growing ecosystems, where the rice seedlings are transplanted into puddled fields and are kept submerged under 5-10 cm of water throughout the growing season, are reliant on rainfall and are subjected to frequent floods and droughts. Around 3000-5000 litres of fresh water is required to produce 1 kg of rice grain (IRRI). With declining fresh water availability, a new concept of aerobic rice production has been developed to grow high yielding lowland rice varieties in non-puddled, aerated and irrigated soil (Bouman *et al.* 2002). Systematic studies on lowland rice varieties grown under aerobic conditions with 77 % less water than in flooded fields in the dry season showed only 23 % reduction in yield (Bouman *et al.* 2005). The aerobic rice cultivation holds promises for farmers, enabling them to cultivate more than one crop every year with limited water availability without compromising too much on the yield.

Being a tropical crop, rice also serves as a host for many diseases and pests, 54 in temperate zones and about 500 in tropical countries (Papademetrieu 2009). Of the

known rice pathogens and pests, the rice blast fungus and root-knot nematodes cause severe damage to rice cultivation in all rice growing ecosystems, especially in the aerobic rice cultivation (Farooq *et al.* 2011; Kreye *et al.* 2009).

The model dicot plant, Arabidopsis, has been the first choice of plant scientists because of its short life-cycle, ease of growth and maintenance, sequenced genome, rich genetic resources and the ease of manipulation (Sijmons *et al.* 1991). Although the studies performed in Arabidopsis have enhanced our knowledge and understanding of the basic plant mechanisms and their responses to various stimuli, monocotyledon plants do not always respond in the same way. The monocotyledons and the dicotyledons diverged 200 million years ago and are significantly diverse in many aspects of development. Thus, it is essential to also study a model monocot crop species. Rice has long been used as the model monocot plant because of its economic and agricultural importance. Availability of the completely sequenced and annotated genome has led to universal acceptance of rice as the model monocot plant. It has a small genome of 420 Mbp with high gene density and also shows high synteny with the other cereals (Goff *et al.* 2002). Since the publication of the draft genome sequences for Nipponbare (japonica cultivar) and 93-11 (indica cultivar) in 2002, rice genome research has come a long way. The expected end of the International rice functional genomic project in 2020, will accomplish the aim of defining the function of all identified rice genes (Zhang *et al.* 2008; Goff *et al.* 2002; Yu *et al.* 2002).

1.2. Drought stress

At least 23 million hectares of rice growing area, i.e. 20 % of the total rice growing area in Asia, is affected by drought of different intensities, a major factor contributing to low and unstable rice production in this area (Pandey 2009). Drought is a situation where water potential and the turgor of plant leaf cells reduces to a level where normal functions are impaired. It causes stomatal closure and limits gas exchange, reduces water content, turgor, water potential and results in wilting of the plant (Shao *et al.* 2008). Rice is affected by drought stress at each developmental stage in all rice growing ecosystems and the crop responds differently to the drought stress in different life stages (Boonjung and Fukai 1996). Plants deploy drought avoidance mechanisms including leaf rolling, stomatal closure, reduced tillering and accumulation of osmoprotectants to prevent severe damage caused due to drought (Hadiarto and Tran 2011). The effect of

vegetative drought stress in upland rice results in reduced tiller number and reduced panicles, whereas drought stress during the reproductive growth phases cause heavy yield losses (Boonjung and Fukai 1996). Rice cultivars also adopt drought escaping strategies by initiating early heading to escape severe drought at critical reproductive time (Guan *et al.* 2010).

1.2.1. Plant morpho-physiological response towards drought stress

Detrimental effects caused by different abiotic stresses are related to disruption of plant water status in one or the other way. The field drought condition can manifest physiological changes similar to other abiotic stresses like high temperature, disturbed ion intake and nutrient deficiencies in plants (Wang, Vinocur and Altman 2003). Verslues et al. (2006) have comprehensively described the physiological changes and stages of the drought response. Drought is characterised by decreased soil moisture potential and reduced available water for the plant. Plants respond to this initial reduction in soil water potential by reducing the water loss and maximising water absorption at the lower soil water potential to ensure normal cell function. These goals are attained by physiological changes like stomata closure, change in root:shoot growth and increase in root to shoot ratio, thickening of the cuticle and increase in tissue water storage. When the soil water potential reduces further, these drought avoidance mechanisms fail to sustain normal plant growth and the plant has to deploy dehydration avoidance mechanisms. The plants require osmotic strategies to prevent water loss; this can be done by increasing solute concentration in the plant cell and thickening of the plant cell wall. If the soil water potential drops further, the plant has to adopt drought-surviving strategies. During extreme water loss, plants undergo changes to try and protect cellular components from effects of dehydration. This involves production of protective proteins and limitation of damage caused by reactive oxygen species (ROS) by reducing their levels in the cell (Verslues *et al.* 2006). The resurrection plants deploy extreme drought survival strategies. These plants can survive absolute water loss and rehydrate when sufficient water is available (Bewley 1979). The study of desiccation tolerance in resurrection plants and use of dehydration as a method of implementing drought stress in laboratory studies has resulted in major advancements in developing drought tolerance in crop species (Moore *et al.* 2009).

In rice, the morpho-physiological drought responsive traits are used for engineering or screening plants for drought tolerance and resistance. Major drought avoidance traits in rice include adaptations in the root system like increase in root depth, root density, root:shoot ratio and root penetration. The shoot-related traits include leaf rolling and reduced stomatal conductance, osmotic adjustment, altered water use efficiency, retention of green leaf and production of fertile spikelet (Fukai and Cooper 1995). The contribution of these traits towards yield of rice under stress conditions is reviewed by Fukai et al. (1995). Various studies have identified quantitative trait loci associated with the morpho-physiological traits to facilitate breeding programs targeted to improve drought tolerance (Price *et al.* 2002; Price, Young and Tomos 1997).

1.2.2. Model systems to study rice drought response in the laboratory

Studies exploring rice drought tolerance and the physiological effect of drought have been performed in fields during the dry season whereas the molecular responses towards drought have generally been investigated by studies carried out in controlled environments. Air-drying or dehydration has been readily adapted as a model of inducing drought stress across plant species. Although the molecular changes marking dehydration are similar to drought stress, it is not a suitable model to mimic the gradual reduction in soil water potential as encountered by plants in the fields. Apart from air-drying, reduction in water potential of the plant growth medium can be achieved by addition of solutes like mannitol or polyethylene glycol (PEG) (Biswas *et al.* 2002; Rai *et al.* 2011). PEG is a non-ionic long chain polymer; high molecular weight PEG is considered a non-penetrating osmoticum that lowers the osmotic potential of experimental media (Rai *et al.* 2011). The reduction in soil water content results in loss of water from the cell wall as well as from the protoplast of the cell but the low molecular weight solutes like mannitol are absorbed by the plant cell resulting in plasmolysis (Verslues *et al.* 2006). The low molecular weight PEG also gets absorbed by the plants and accumulates in plant tissue resulting in negative effects on the plant growth (Fan and Blake 1997). Although, high molecular weight PEG has been used as an osmoticum in plant systems, it has been difficult to correctly measure the reduction in osmotic potential that it causes (Steuter, Mozafar and Goodin 1981). These methods are advantageous in assuring homogenous stress making it easy to screen large numbers

of plants under similar conditions, but the plant responses to these methods are not similar to those observed in soil.

1.2.3. Plant molecular response towards drought stress

Characterisation of the molecular aspects of the drought response in rice mainly comprises study of regulatory elements like transcription factors or protein kinases along with downstream genes belonging to functional categories involved in metabolism or direct stress tolerance. The main family of transcription factors (TF) associated with drought responses in plants are known as dehydration responsive element binding proteins or DREBs. These proteins have an ethylene responsive binding factor motif (ERF) which binds to the *cis* acting dehydration binding domain (A/GCCGAC) of the various downstream stress responsive genes (Matsukura *et al.* 2010). The *DREB* genes can be divided into two functional categories, *DREB1* and *DREB2*. A total of six *DREB2* genes have been identified in rice, *DREB2A, DREB2B, DREB2C, DREB2D, DREB2E* and *osABI4* (orthologous to the *DREB2*s). Out of these, *DREB2B,* plays an important role in dehydration stress induced by air-drying. Two alternatively spliced forms of *DREB2B* are identified, *DREB2B1* which is constitutively expressed and *DREB2B2,* which is alternatively spliced from *DREB2B* under stress conditions (Matsukura *et al.* 2010). Transgenic rice plants over-expressing different *DREBs* have successfully shown improved abiotic stress tolerance in laboratory conditions. *DREB2A* over-expressing plants show improved salt and dehydration tolerance when the stresses are imposed using mannitol and NaCl respectively (Mallikarjuna *et al.* 2011). Transgenic over-expression of *DREB1F* enhances tolerance to dehydration stress induced by PEG 6000 and air-drying in both rice and Arabidopsis (Wang *et al.* 2008). The transgenic rice over-expressing *DREB2B2, DREB1G* and *DREB1E* show improved rate of survival under water deficit in a soil-based screening system when compared to the wild-type plants (Chen *et al.* 2008a).

Another family of transcription regulators involved in plant stress response is the *NAC* (*NAM, ATAF* and *CUC*) family of genes, which is specific to the plant kingdom. The NAC family members are transcription factors and affect the expression of many downstream abiotic stress-responsive genes. The members of this family can form homodimers and heterodimers. They also interact with other regulatory elements to generate abiotic and biotic stress responses. The NAC proteins contain a conserved N-

terminus and a DNA binding domain. The spatial distribution and role of rice NAC family members under biotic and abiotic stresses have been detailed elsewhere (Fang *et al.* 2008; Nuruzzaman *et al.* 2010) Transgenic rice over-expressing *OsNAC9* and *OsNAC10* genes display enhanced drought tolerance through improved root characteristics, resulting in improved yield potential in the field (Redillas *et al.* 2012a; Jeong *et al.* 2010). Over-expression of *OsNAC52* in Arabidopsis makes the plants sensitive to ABA and improves drought tolerance (Gao *et al.* 2010). The members of the NAC family are also involved in biotic stress responses and auxin signalling in plants (Sun *et al.* 2013; Zheng *et al.* 2009). Transgenic rice plants constitutively overexpressing *OsNAC6* show increased tolerance to biotic and abiotic stresses but with negative influence on growth and yield (Nakashima *et al.* 2007; Ohnishi *et al.* 2005). *OsNAC5* directly controls the expression of other stress-induced genes like Late Embryogenesis Abundant (*LEA*), it also confers drought tolerance on transgenic rice plants but causes a negative effect on the plant growth (Takasaki *et al.* 2010).

Drought responsive physiological changes in rice are a consequence of overlapping signalling pathways. The stomatal closure and increased sensitivity to ABA in the guard cells that leads to drought resistance in rice is caused by strong expression of a *SNAC1* transcription factor (Hu *et al.* 2006). A zinc finger transcription factor, also known as Drought and salt tolerance (*DST*), negatively regulates stomatal closure by H_2O_2-mediated signalling (Huang *et al.* 2009c). The recent identification of the *OsSRO1c* gene has linked these two pathways of stomatal closure under drought stress. *OsSRO1c* is regulated directly by the *SNAC1* gene and its over-expression leads to down regulation of *DST* and accumulation of H_2O_2 resulting in stomatal closure. The *OsSRO1c* induced stomatal closure is independent of ABA but its protein product interacts with the OsDREB2B TF (You *et al.* 2013).

Other members of TF families APETELA2, bZIP, zinc finger MYB and WRKY have also been characterised and are known to play important roles in drought responsiveness in rice (Hadiarto and Tran 2011; Todaka *et al.* 2012; Agarwal and Jha 2010).

Apart from the TFs, drought also influences expression of various protein kinases (PK) involved in signalling cascades. The receptor-like protein kinase, *OsSIK1,* provides increased drought tolerance by activation of the oxidative system. Over-expression of the *OsSIK1* gene leads to drought tolerance and activation of downstream genes like peroxidases, superoxide dismutase and catalase that reduce the accumulation of H_2O_2 in

the leaves and also affect the stomatal density (Ouyang *et al.* 2010). The mitogen-activated protein kinase (MAPK) signal cascade is the most common signalling cascade involved in plant hormone response, development, cell division and abiotic stress responses. It consists of a three kinase system MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK. The role of MAPK in plant defence responses are well characterised (Agrawal, Iwahashi and Rakwal 2003; Zhang and Klessig 2001). Of the known MAPKs in rice, *MAPK5, MAPK7, MAPK8* and *MAPK12* are known to be drought induced and *MAPK4* is repressed under drought stress (Hadiarto and Tran 2011). In rice, a Raf-like MAPK identified from a drought-hypersensitive mutant (*dsm1*) enhances drought and salt tolerance by altering ROS scavenging mechanisms (Ning *et al.* 2010). The MAPK kinases regulate the expression of downstream genes, mostly transcription factors like *OsWRKY30* and enhance drought tolerance (Shen *et al.* 2012). Apart from the ROS scavenging mechanisms, the MAPK cascade also enhances drought tolerance via an ABA-mediated stress response (Singh and Jwa 2013).

Change in Ca^{2+} flux in a cell is perceived by members of the calcium-dependent protein kinase (CDPK) and calcineurin B-like interacting protein kinase (CIPK) families of protein kinases. Of the CDPKs, *OsCDPK7* is localised in rice tissues where water stress occurs most severely (vascular tissue of roots) and enhances the expression of the putative hydrophilic protein producing gene *rab16A*, potentially involved in water retention and improved drought tolerance (Saijo *et al.* 2001b; Saijo *et al.* 2001a). The CIPKs that are known to be drought responsive in rice, also play an important role in plant development and reproduction. *OsCIPK31* is required for germination and seedling development but also modulates expression of stress responsive genes (Piao *et al.* 2010). *OsCIPK23* regulates expression of various drought-responsive genes and is also required for pollination (Yang *et al.* 2008). The *OsCIPK12* over-expression line has an increased concentration of proline and soluble sugars that might impart improved drought tolerance.

Another drought responsive family of PKs is stress activated protein kinases *(SAPK)* belonging to the superfamily of sucrose non-fermenting related kinases (SnRK). There are ten *SAPK* genes known in rice and all of them respond to hyperosmotic stress. *SAPK8, SAPK9* and *SAPK10* also respond to ABA and regulate the expression of bZIP transcription factors (Goodell and Ferris 1989).

At low soil water potential, plants accumulate osmoprotectants that maintain the osmotic balance of the cell and protect the cell membrane from the devastating effects of reduced water potential. These osmoprotectants include non-reducing sugars like trehalose, certain amino acids like proline and glycine and the fully N-methylated amino acid derivatives like betains (Rontein, Basset and Hanson 2002). The genes involved in biosynthetic pathways of these solutes are downstream targets of the TFs and PKs induced by drought. Accumulation of osmoprotectants by modifying metabolic enzymes involved in production of these molecules enhances drought tolerance in rice (Redillas *et al.* 2012b; Li *et al.* 2011).

Drought can lead to mis-folding of RNA and degradation of proteins. Thus, a major category of genes affected by drought include the heat shock protein (HSP) chaperones and other protective proteins. Rice has a total of 74 HSPs classified into four categories; sHSP, HSP70, HSP90 and HSP100 (Xiang *et al.* 2013). These genes are activated by factors (HSFs) that bind to the heat shock element of these genes. Rice has 25 *HSF* genes, all of which respond to ABA whereas only four are drought-specific (Chauhan *et al.* 2011). Another class of molecular chaperone proteins called late embryogenesis abundant (LEA) proteins are also produced in response to drought. The LEAs are hydrophilic proteins and are found abundantly in germinating seeds in unstressed conditions. Their putative role in drought stress is to maintain structure of the cell membrane and ion balance (Ashraf 2010). The expression levels of rice *LEA* genes increase under water deficit. Over-expression of *OsLEA6* and *OsLEA3-1* has been successfully used to enhance drought tolerance of rice plants in the field (Xiao *et al.* 2007; Rodríguez-Valentín *et al.* 2013).

Last but not the least, drought influences biosynthesis of phytohormones; specifically the abiotic stress response is mediated by ABA. Water deficit alters expression of enzymes involved in biosynthesis of ABA, increasing its production that results in ABA-mediated drought response. Drought induces expression of a GH3 family gene, *OsGH3-2,* that catalyses conjugation of indole acetic acid (auxin) to amino acids and modulates the level of ABA in response to stress (Du *et al.* 2012). The drought response in plants can be classified as ABA-dependent or ABA-independent (Cutler *et al.* 2010; Agarwal and Jha 2010).

Altogether, ABA, TFs and PKs modulate a vast network of signalling cascades directing expression of downstream genes in response to drought stress in rice. Some

parts of this complex network are well studied (like the ABA-dependent drought response) whereas others still need exploring (like the role of LEAs).

1.3. Rice nematode problems

Plant parasitic nematodes (PPN) account for an annual agricultural loss of around $157 billion, globally (Abad *et al.* 2008). It is estimated that half of that total cost is derived from the losses in just two crops, rice and maize, with rice suffering a total loss of $35 billion annually (Berg and Taylor 2009). A total of 200 PPN species have been reported to be associated with rice, out of these the *Meloidogyne* spp (root-knot nematodes - RKN) and *Heterodera spp* (cyst nematodes - CN) are the predominant root pests. Major RKN species infecting rice include *M. graminicola*, *M. incognita*, *M. oryzae*, *M. javanica* and *M. arenaria*. They are cosmopolitan, affecting rice plants in all rice ecosystems in various countries across the globe. The four main cyst nematode species infecting rice are *H. oryzicola*, *H. elachista*, *H. oryzae* and *H. sacchari.* They are localised in certain parts of the world (Bridge, Luc and Plowright 1990). *Meloidogyne spp* have a broad host range, they can reproduce on cereals, vegetable and weeds, whereas the host range for rice CNs is narrow and excludes many wild and cultivated Gramineae members (Bridge, Luc and Plowright 1990).

Out of all the nematode pests of rice, *M. graminicola*, Golden and Bridge is considered the most serious pest in all ecosystems (Roberts 2007) . It was traditionally reported in the fields of Laos, Vietnam, Burma, Thailand, Philippines, India and USA. In the last few years, it has been reported to be widespread in rice and wheat growing regions of Nepal, Pakistan and the majority of upland rice growing regions of India. It can affect lowland rice, upland rice, rice in nurseries and even in deep water production systems (Pokharel *et al.* 2007). The severity of *M. graminicola* infection is dependent on the initial nematode density in soil along with the soil moisture and flooding regimes (Poudyal *et al.* 2005; Soriano, Prot and Matias 2000). The young rice grown in drained soil is more susceptible to infection; 2nd-stage juvenile nematodes penetrate the root within five hours after artificial inoculation and many juveniles can enter the same root tip. The eggs stay viable in moist and water-logged soil for as long as 12 months. The life cycle takes 26-51 days, depending upon the time of the year (MacGowan and Industry 1989). The rice varieties under upland conditions suffer a 23.6 % of yield loss

and susceptible varieties show significant reduction in biomass, both fresh and dry, along with reduced grain filling (De Waele *et al.* 2013).

Of the CNs, *H. sacchari* is a problem on upland and flooded rice and sugarcane throughout West Africa and some parts of Asia (Coyne, Smith and Plowright 2001). The yield losses in Asia due to *H. sacchari* are not well documented while in Côte d' Ivoire, intense wet season rice cropping has increased *H. sacchari* populations leading to 50 % yield losses (Bridge, Plowright and Peng 2005).

1.3.1. Lifecycle of the root-knot nematodes

The RKNs are obligate, sedentary endoparasites that can manipulate the host plant functions to suit their own benefits. The RKN juveniles undergo the first moult in the egg and the second stage juveniles (J2s) hatch out of the eggs when the conditions are favourable. Hatching of *Meloidogyne* juveniles is dependent on the soil moisture, soil aeration and temperature (Wallace 1968; Goodell and Ferris 1989). Although, hatching in *Meloidogyne* does not require host root exudates and can take place in just water, the root exudates affect rate of hatching and percentage hatch in some species (Curtis, Robinson and Perry 2009). The movement of *Meloidogyne* juveniles in soil requires ideal moisture and pore size (Prot and Van Gundy 1981; Eo *et al.* 2007). The pre-parasitic J2s infect the plants near root tips. The nematode mechanically enters the plant using the protracting stylet, supported by the secretion of cell wall degrading enzymes (Davis, Hussey and Baum 2004). The juveniles travel intercellularly between the cortical cells towards the root tip. On reaching the root tip they take a U-turn to enter the vascular tissue and start feeding. At this stage, the juveniles become sedentary and parasitic. They induce formation of feeding sites by re-differentiating parenchymatic cells into multinucleated and hypertrophied feeding cells also known as giant cells (Wyss, Grundler and Munch 1992; Wyss and Grundler 1992). The cells surrounding the giant cells undergo a few rounds of cell division resulting in formation of a gall. These giant cells act as specialised nutrient sinks that support the growth and reproduction of the nematode (Kyndt *et al.* 2013). The J2 feeds from the giant cells then undergoes three subsequent moults, without feeding, to reach the mature adult stage. RKN are dioecious and gonochoristic, with separate, morphologically distinct males and females. Most species are obligate or facultative parthenogenetic, whereas very few are obligatorily amphimictic (Bird *et al.* 2009). The adult vermiform males emerge from the cuticle and

leave the gall after the fourth moult. Unlike J2s and females, males do not feed. All the energy required for further development and survival was obtained while it was a J2. The males are common in species that are amphimictic but rare in the parthenogenetic species. The females become pear shaped and remain sedentary after the fourth moult. They continue feeding on the giant cells for the remainder of the lifecycle. Eggs are deposited through the vulva into a gelatinous matrix, called egg mass, that is generally on the root surface (Eisenback 1985) (Figure 1-1).

M. graminicola exhibits a temperature-dependent lifecycle on rice, lasting for 26-51 days depending on the time of the year (Fernandez, Cabasan and De Waele 2013; MacGowan 1989). It is classified as meiotic parthenogenetic species, with occasional cross fertilisation (Triantaphyllou 1969; Dutta *et al.* 2011) .

1.3.2. Life cycle of cyst nematodes

The life cycle of CNs is similar to that of RKNs but differs in the method of infection and feeding. The CN J2s infect plant roots in the zone of elongation with the help of stylet thrusts and a cocktail of cell wall degrading enzymes (Wyss, Grundler and Munch 1992). The J2s migrate intracellularly towards the vascular bundle, in a more destructive fashion than the RKN J2s. The J2 rapidly and vigorously probes its stylet in numerous cells and punctures them while moving towards the vascular bundle; the protoplast of the punctured cells collapses immediately marking the nematode migratory path by a number of dead cells. The destructive migratory behaviour of the J2 continues until it identifies a procambial or pericycle cell whose protoplast doesn't collapse and responds favourably to the probing. This cell is selected as the initial syncytial cell or ISC (Sobczak and Golinowski 2011). The J2 stops moving and enters the sedentary phase. After a preparatory phase of 7 hrs, secretions from the nematode's oesophageal glands are injected into the ISC and the nematode starts feeding from this cell. The syncytium expands by incorporating adjacent procambial cells and the pericycle cells. This proceeds first by increased distribution of plasmodesmata, and widening of pre-existing plasmodesmata (Grundler, Sobczak and Golinowski 1998), followed by partial cell wall dissolution and fusion of neighbouring protoplasts. A syncytium associated with an adult female can include 200 cells and reaches its maximum size at 10 days post infection (dpi) (Hussey *et al.* 1998; Urwin *et al.* 1997b). Like RKN, the syncytium is the only source of nutrients for the feeding nematode. The CNs are dioecious and

gonochoristic, with separate, morphologically distinct males and females. The sex of the undifferentiated second stage juvenile is determined by environmental factors and the quality of the nutrient supply provided by the developing syncytium (Grundler, Betka and Wyss 1991; Trudgill 1967). The J2 retracts its stylet, stops feeding and moults to J3. After the moult, the J3 starts feeding again and continues to feed for several days until it becomes a swollen fusiform. The J3 stage ends with another moult to J4, after this moult the male stops feeding and after another moult the adult male attains vermiform state and becomes motile. A syncytium induced in the pericycle and associated with the male juvenile shows growth abnormality. Whereas, the J4 female remains sedentary and continues to feed from the syncytium, it undergoes another moult resulting in a spherical or lemon-shaped adult female nematode that further continues to feed from the syncytium (Sobczak and Golinowski 2011; Sobczak and Golinowski 2009). CNs reproduce by obligatory amphimixis, the adult males fertilise females and then die. The female starts laying eggs and once all the eggs are laid, the female dies too. The body wall of the dead female nematode starts tanning and hardening, enclosing most of the eggs inside it (Figure 1-2). It is now called a cyst. The unhatched eggs can stay viable inside the cyst for several years (Kaushal and Sharma 1999). They start hatching on receiving an optimum hatching stimulus from a host plant root system; cysts of some species can also hatch in water (Clarke and Perry 1977). The rice CNs take 24-30 days to complete one life cycle and thus there are several generations during the host growing season (Ibrahim *et al.* 1993).

1.3.3. Plant response towards nematode infection

Plant parasitic nematode infection causes local tissue damage and is characterised by reduction in photosynthetic rate at a high level of infestation (Bird 1974; Loveys and Bird 1973). In initial phases, a low level of infection can enhance plant growth, but the growth gradually decreases as the infection progresses (Barker and Olthof 1976). Nematodes can also result in reduction of nutrient components (potassium, zinc and magnesium) and increase in concentration of calcium in plants (Melakeberhan *et al.* 1985; Melakeberhan *et al.* 1987). The root-knot nematode, *M. ethiopica* influences the plant water status and reduces the stomatal conductivity, transpiration and photosynthetic rate by up to 60 % in tomato plants (Strajnar *et al.* 2012). *M. incognita*

also reduces the chlorophyll content and carotenoid content of sugar beet genotypes (Korayem *et al.* 2012).

The molecular events following nematode invasion in a plant are targeted to suppress host defences to sustain a feeding site and also to protect the nematode from direct plant attack. As an immediate response to the parasite invasion, the plant generates a quick and lethal response that includes killing of plant cells to kill the parasite, this response is called the hypersensitive response. The hypersensitive response is initiated by recognition of unique pathogen molecular signatures presented by the pathogen on the plant surface known as the pathogen-associated molecular patterns (PAMPs). The immune response initiated in plants by recognition of PAMPs is termed PAMP-triggered immunity (PTI). Pathogens have developed mechanisms to successfully suppress plant PTI which has led to evolution of novel immune responses in plants capable of activating effector-triggered immunity (ETI) (Jones and Dangl 2006). The activation and suppression of PTI and ETI responses in nematode-plant interactions are not well comprehended. The initial phase of hypersensitive response due to PTI and ETI includes generation of ROS, change in intracellular redox state and calcium ion concentration. These changes lead to activation of signalling cascades that result in transcription of defence related genes (pathogenesis related proteins and key enzymes required for biosynthesis of secondary metabolites). This hypersensitive reaction can also result in a large scale programmed cell death around the feeding site (Smant and Jones 2011). The plant defence system also directly attacks the nematode by generation of ROS and compounds (like terpenoids) that have broad spectrum anti-pathogen effects. Although the mechanisms by which the nematode inactivates these nematicidal compounds are not well known, it is suggested that the nematode produces enzymes like glutathione-S-transferase (secreted in the host roots by *M. incognita*) that are capable of inactivating such compounds (Dubreuil *et al.* 2007). The PPN are well equipped to deal with ROS generated by the host, *G. rostochiensis* produces glutathione peroxidase that metabolises hydrogen peroxide (Jones *et al.* 2004). *M. incognita* expresses peroxiredoxins that fine tune the redox state at the plant pathogen interface and help the nematode in establishing a successful infection (Dubreuil *et al.* 2011). During an incompatible plant nematode interaction, nematode effectors or avirulence factors (AVR) are identified by the plant's resistance genes (*R*). This subsequently leads to activation of host signal transduction pathways resulting in unfavourable conditions

for survival of the nematode (Williamson and Kumar 2006). During a compatible interaction, the nematode successfully subdues the plant's defence response and establishes a feeding site. Mi-CRT, a calreticulin secreted by RKN, is a good example of a nematode effector that can suppress PTI during a compatible interaction (Jaouannet *et al.* 2013).

Phytohormones play an important role in successful nematode parasitism on the plant. Auxin is necessary for establishment of nematode infection. PPNs induce strong expression of the synthetic auxin-responsive promoter DR5 during the early stages of feeding site development (Karczmarek *et al.* 2004). Tomato and Arabidopsis plants with defects in auxin signalling support a reduced number of developing cysts. The cyst nematode can subvert the auxin distribution network to facilitate the infection (Grunewald *et al.* 2009). The Hs19C07 effector from *H. schachtii* interacts with the Arabidopsis auxin influx transporter LAX3 and modulates auxin flow in the root cells and causes hydrolysis of the cell walls for syncytium development (Lee *et al.* 2011a). Formation of a feeding site by PPN includes re-entry of the cell into the cell cycle and the cytokinins that regulate the cell cycle are presumed to be involved in this process. In *Lotus japonica* infected by RKN, high levels of the cytokinin responsive promoter *ARR5* is seen when the juveniles reach the differentiating vascular bundle (Kyndt *et al.* 2013). Apart from the plant endogenous cytokinin, both RKN and CN may produce cytokinins that can aid activation of the cell cycle for development of a feeding site (De Meutter *et al.* 2003).

ETI and PTI also prime activation of the salicylic acid (SA), jasmonic acid (JA) and ethylene-mediated hormonal signalling pathways. Biotrophic parasites are known to induce SA, whereas necrotophic parasites and wounding initiate the JA and ethylene response in plants. Hormonal signalling induces systemic acquired resistance (SAR) in the non-infected plant parts. In dicots, salicylic acid is known to induce SAR in the plant, leading to systemic expression of anti-microbial pathogenesis related (PR) genes (Durrant and Dong 2004). Induction of the *PR1, PR2* and *PR5* genes is used as a marker of SA-induced SAR whilst the *PR3* and *PR4* genes are typically induced by the JA-dependent response (Molinari, Fanelli and Leonetti 2013). Rice has particularly high endogenous levels of SA and the mutants deficient in SA content are highly susceptible to pathogen attack (Balmer, Planchamp and Mauch-Mani 2013). In dicot plants, the vital role of SA in SAR is well studied and understood but its role in monocots is not

well known. Systemic response of rice towards nematode infection is a multi-layered complex interaction between various hormonal pathways. Exogenous application of ET (ethephon) and JA in rice shoots increases the expression of the *PR1b* and *PR1a* genes in the roots, but application of SA does not cause any change in expression of these genes. The ET-mediated defence response in rice requires an intact JA-mediated response pathway whereas the JA-mediated response pathway is independent of ET (Nahar *et al.* 2011). Altogether, intact SA, JA and ET pathways are required for an effective basal defence response to nematodes in rice. This response is negatively influenced by the ABA pathway, which is known to function antagonistically to the SA, JA and ET pathways (Nahar *et al.* 2012). The JA pathway is also negatively regulated by the growth hormone brassinosteroid (BR) pathway, wherein exogenous application of a high concentration of BR in the shoots of rice plants decreases BR production in roots and increases JA production resulting in resistance to RKN (Nahar *et al.* 2013).

The molecular response of rice towards PPN has, until now, been elucidated by exogenous application of hormones or by studying young rice seedlings infected with 200-400 J2s each in tissue culture. The model used in most of these studies is not realistic and is driven by one or the other variable. Systematic studies exploring the molecular response of rice plants in vegetative and reproductive stages towards realistic nematode infection conditions as encountered by plants in fields are required to put together pieces of this complex puzzle.

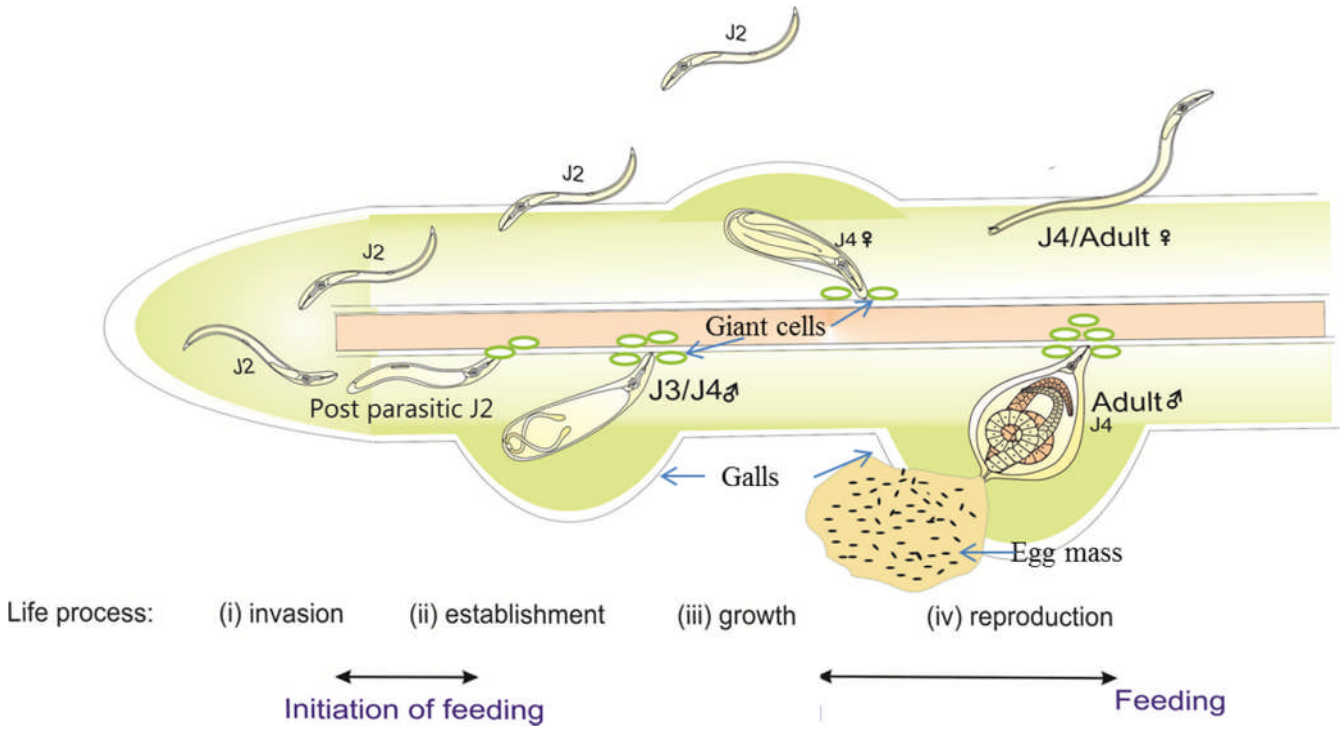

Figure 1-1 Life cycle of a root-knot nematode

The second stage root-knot nematode juvenile (J2) enters plant root behind the root tip, it migrated towards the root tip intercellularly and then takes a U-turn and initiates a feeding site in the vascular bundle and becomes sedentary. It induces re-differentiation of the parenchyma cells into nutrient sinks called giant cells. The cells surrounding giant cells undergo several rounds of cell division to form galls. The J2 undergoes two subsequent moults without feeding to form third stage juvenile (J3) and fourth stage juvenile (J4). The adult vermiform males emerge from the cuticle and leave the gall after the fourth moult. Unlike J2s and females, males do not feed. The females become pear shaped and remain sedentary after the fourth moult. They continue feeding on the giant cells for the remainder of the lifecycle. Eggs are deposited through the vulva into a gelatinous matrix, called egg mass, that is generally on the root surface.

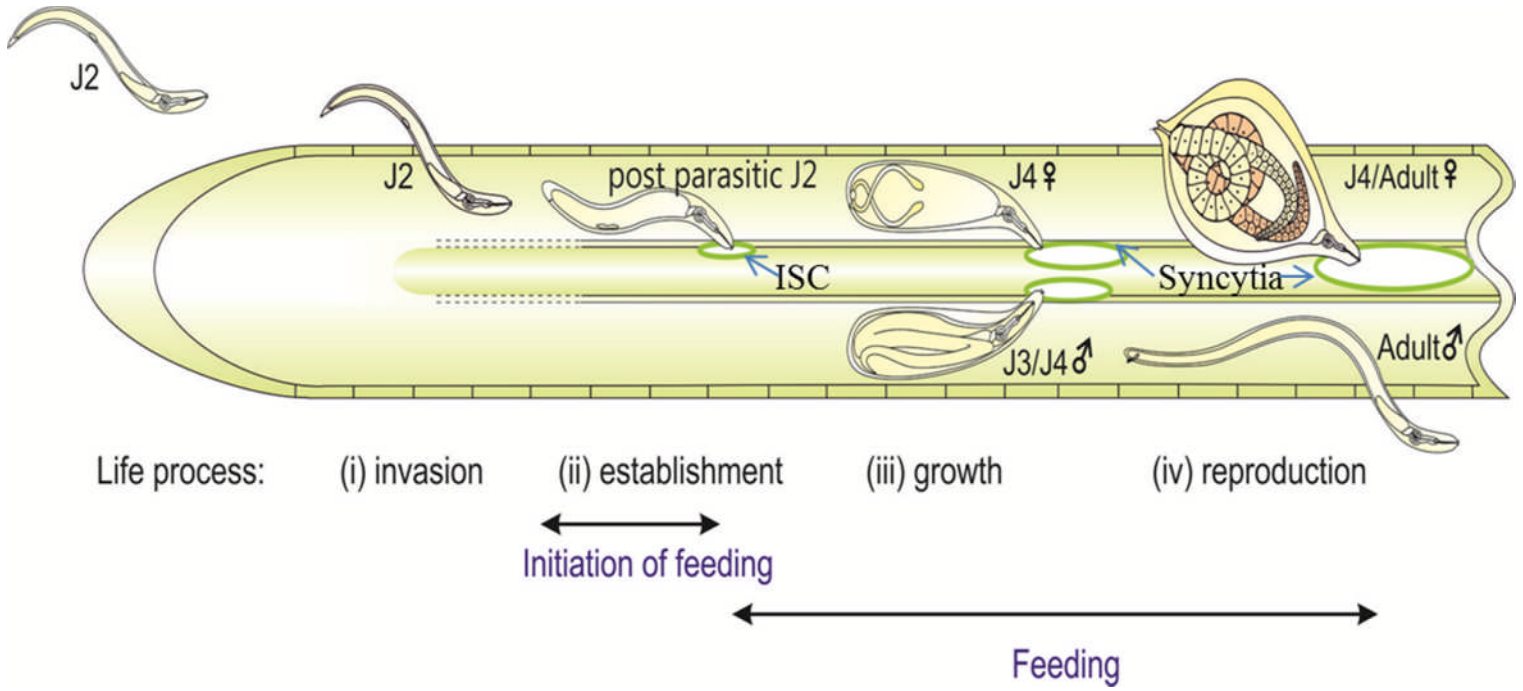

Figure 1-1 Life cycle of a cyst nematode

The second stage cyst nematode juvenile (J2) enters the plant root in the zone of elongation, migrates intracellularly towards the vascular bundle where it selects a procambial or pericycle cell as the Initial Syncytial Cell (ISC). The J2 retracts its stylet, stops feeding and moults to J3. After the moult, the J3 starts feeding again and continues to feed for several days until it becomes a swollen fusiform. The J3 stage ends with another moult to J4, after this moult the male stops feeding and after another moult the adult male attains vermiform and becomes motile. Whereas, the J4 female remains sedentary and continues to feed from the syncytium, it undergoes another moult resulting in a spherical or lemon-shaped adult female nematode that further continues to feed from the syncytium. The female starts laying eggs and once all the eggs are laid, the female dies too. The body wall of the dead female nematode starts tanning and hardening, enclosing most of the eggs inside it.

1.4. Simultaneous stresses experienced by plants in the field

Traditional molecular studies designed to explore plant stress responses have been driven by systems that artificially impose one particular stress or exogenous application of hormones on model plant species grown in unrealistic laboratory conditions. The results of such studies have enhanced our understanding of the signalling cascades and hormonal pathways that mediate plant responses towards various stresses and have been used in achieving tolerance to biotic and abiotic stresses. But time and again, the plants engineered for tolerance to a single biotic or abiotic stress in the laboratory have failed to attain similar results in the fields (Atkinson and Urwin 2012; Mittler 2006). This is because the crops in the field encounter more than one type of stress at any given point in time and with the prophesied climate change model, incidences of simultaneous biotic and abiotic stresses on plants are bound to increase. The effect of climate change on plant-pest interactions has been widely reviewed in recent years (Garrett *et al.* 2006; Chakraborty 2005; Scherm 2004; Gregory *et al.* 2009; Newton, Johnson and Gregory 2011; Luck *et al.* 2011). The response of plants to the combination of biotic and abiotic stresses is custom-tailored for the nature of the stresses and there can be additive, negative or interactive effects of the responses initiated by any of the stresses individually (Atkinson and Urwin 2012). Evidence suggests that increased carbon dioxide levels in the atmosphere will lead to suppression of plant defence responses by manipulation of the hormonal signalling pathways in plants. Soybean plants show down-regulation of JA and ET pathways resulting in reduction of cysteine protease inhibitors under increased CO_2 levels which in turn reduces the plants' defence against coleopteran pathogens (Zavala *et al.* 2008). At the same time, the increased CO_2 levels also result in the increased global expression of SA in soybean plants (Casteel *et al.* 2012). The increased CO_2 levels are likely to provide legumes with a photosynthetic advantage and protection against drought-induced loss in N_2 (Rogers, Ainsworth and Leakey 2009). In tomato plants, elevated CO_2 levels have resulted in decreased resistance to the RKN, *M. incognita* (Sun *et al.* 2010). Apart from elevated levels of CO_2, temperature plays an important role in plant pathogen interactions (Zhu, Qian and Hua 2010; Fu *et al.* 2009). Temperature-dependent resistance is seen in rice towards blast disease, towards broomrape in sunflower and clover, towards downy mildew in musk melon and towards stripe rust in wheat (Webb *et al.* 2010; Eizenberg *et al.* 2009; Balass, Cohen and Bar-Joseph 1993; Fu *et al.* 2009; Eizenberg, Colquhoun and

Mallory-Smith 2009). Increase in temperature will also lead to more rapid development, increased reproductive potential and more generations of pests and pathogens in a season. These changes in pest lifecycle and productivity are going to cause unprecedented damage to the crops in one season (Scherm 2004). Drought can aid pest and pathogen outbreaks in fields, at the same time pathogens can severely influence plant water relations and lead to low water potential in plant cells (Mattson and Haack 1987). The bacterium *Xylella fastidiosa* causes pathogen-induced drought in grape by severe reduction of water potential (Choi *et al.* 2013). In the case of foliar pathogens, stomatal closure is the first physiological barrier in the defence response. Stomatal closure is also a drought avoidance strategy, thus drought-induced stomatal closure reduces pathogen entry into the plant tissue. Similarly, pathogen-induced stomatal closure helps the plant in efficient use of water (Sawinski *et al.* 2013). Drought enhances the symptoms of fungal charcoal rot disease in common bean (Mayek-Perez *et al.* 2002). Drought leads to reduction in plant water status causing concentration of metabolites in the plant tissue. Increased concentration of defence compounds in drought-stressed tomato plants results in reduced susceptibility towards the herbivore *Spodoptera exigua* (English-Loeb, Stout and Duffey 1997). But change in herbivore feeding behaviour also depends on the nature of the pest and its specificity towards the plant species (Gutbrodt, Mody and Dorn 2011). Drought stress can also influence the interaction between two pathogens acting on the same plant and *vice versa*. Root-feeding herbivores can also enhance resistance against foliar herbivores by ABA-mediated hydraulic changes (Erb *et al.* 2011). The plant response towards simultaneous infestation by a foliar herbivore (aphids), their parasitoids and a root herbivore is also altered by drought stress (Tariq *et al.* 2013). Drought-induced changes in roots can interact or counteract root-specific pathogens. In water dependent agricultural ecosystems, drought can increase the incidence of soil borne disease, especially PPNs. Drought and PPN infection are two biotic and abiotic stresses that are often encountered simultaneously by rice plants in fields. Drought can increase susceptibility of rice to RKN infection in all ecosystems, especially in aerobic rice cultivation. Cyst nematodes can contribute to the drought-related losses in rice by causing reduced stomatal conductance and reduced leaf water potential (Audebert *et al.* 2000). A study on simultaneous drought and CN infection on Arabidopsis has revealed

that under simultaneous biotic and abiotic stress, the plant responses are dominated by abiotic stress-responsive changes (Atkinson, Lilley and Urwin 2013).

1.4.1. Molecular events in simultaneous biotic and abiotic stress

Current investigations of biotic and abiotic stress responsive pathways in plants suggest that there are significant convergence points between them. Beyond signal perception, stress responses in both biotic and abiotic stresses remain a complex web of interactions between secondary messengers, protein kinases and transcription factors. Differential regulation and interaction of these elements finally results in expression of downstream stress-responsive genes, which characterises the physiological response of a plant towards the stimuli. Recent studies have proved that the plant response to pairs of different biotic and abiotic stress is individually tailored and dependent on the nature of the stress and plant species (Atkinson and Urwin 2012). The general cross-talk between biotic and abiotic stress has been formulated based on the knowledge gained from the response of individual stresses, it's been summarised and reviewed (Atkinson and Urwin 2012; Fujita *et al.* 2006; Mittler 2006).

Of the stress responsive TFs, the members of WRKY family of TFs are responsive to both biotic and abiotic stress and play a vital role in fine-tuning the plants' response to simultaneous stress. In rice, *WRKY13* antagonistically regulates the response to drought and bacterial disease by selectively binding to the *cis*-acting elements and specific sequences in the promoters of *SNAC1* and *WRKY45-1*. It can also auto-regulate its own expression by binding to the promoter (Xiao *et al.* 2013). *WRKY45* imparts resistance against the fungal and bacterial pathogens in rice by differential mechanisms (Shimono *et al.* 2012). The *WRKY45-1* allele negatively regulates ABA signalling and also increases plant susceptibility to bacterial pathogens. The *WRKY45-2* allele positively regulates ABA signalling and increases resistance to bacterial pathogens (Tao *et al.* 2011). But both alleles positively regulate resistance to fungal blast disease (Tao *et al.* 2009). *WRKY76* antagonistically regulates the response of rice to blast disease and cold stress (Yokotani *et al.* 2013b). *WRKY82* enhances defence against biotic pathogens and tolerance against abiotic stress via the JA /ET pathways (Peng *et al.* 2011).

Different disease resistant cultivars have varying natural expression of *OsMYB4* leading to varying degrees of disease resistance to sheath blight and leaf blight diseases in rice

(Singh *et al.* 2013). Ectopic expression of the rice *OsMYB4* TF enhances abiotic and biotic stress tolerance in many plants including Arabidopsis, tomato and apple (Vannini *et al.* 2007; Pasquali *et al.* 2008; Vannini *et al.* 2006). The JA-induced *MYB* gene, *JAmyb,* is induced by high salinity, osmotic stress and ROS and its over-expression results in induction of JA-induced transcription factors that play an important role in biotic stress response (Yokotani *et al.* 2013a).

The *OsNAC6* gene acts as a transcription inducer for biotic and abiotic stress responses in rice. Constitutive over-expression of *OsNAC6* results in increased tolerance to dehydration and salt stress along with increased resistance against blast disease but with growth and yield penalty (Nakashima *et al.* 2007). *OsNAC5* also enhances abiotic stress tolerance in rice and is responsive to JA but doesn't cause any negative effect on plant growth (Takasaki *et al.* 2010). A plant-specific TF family, ethylene responsive factor TFs, bind to the GGC sequence specifically found in the *PR* genes. These TFs are mainly involved in abiotic stress responses in plants. Four ethylene responsive genes, *BIERF1-4,* are up-regulated by salt, drought, wounding and fungal pathogens (Cao *et al.* 2006).

Apart from the TFs, various protein kinases also act as the convergence points in biotic and abiotic stress pathways in rice. Out of the 17 known rice MAPK genes, five are induced by both biotic and abiotic stresses (Rohila and Yang 2007). *OsMAPK5* is the most studied rice MAPK; it renders ABA-mediated tolerance in abiotic stress and resistance to brown spot and negatively regulates the response to rice blast fungus (Sharma *et al.* 2013). Members of the rice CDPK family are also involved in cross-talk between biotic and abiotic stresses. OsCDPK12 regulates genes involved in ROS scavenging in stressed plant cells resulting in reduced accumulation of H_2O_2. Over-expression of *OsCDPK12* leads to positive regulation of salt tolerance and negative regulation of blast resistance (Asano *et al.* 2012). *OsCDPK13* is involved in the gibberellic acid-mediated response in rice leaf sheath and cold tolerance (Abbasi *et al.* 2004). Four CIPK PKs (*OsCIPK 2*, *OsCIPK 10*, *OsCIPK 11* and *OsCIPK 14*) also play important roles in the cross-talk between biotic and abiotic stresses (Chen *et al.* 2011b). Another family of PKs, known as dual specificity PKs (*OsDPK),* also show response to biotic and abiotic stresses. *OsDPK1*, *OsDPK2* and *OsDPK3* are all induced by exogenous application of ABA, drought, salinity and in response to the rice blast fungus (Gu *et al.* 2005).

1.5. Engineering crops for food security

To attain food security for the predicted 9 billion humans, crop yield and nutrition value has to be improved. Considering the predicted climate change, it is necessary to develop crop varieties with improved performance under combinations of biotic and abiotic stresses.

The knowledge obtained from molecular studies exploring plant responses towards various biotic and abiotic stresses has been used to engineer transgenic crops with improved stress management to improve plant yield (Tester and Langridge 2010; Peleg *et al.* 2011; Mittler and Blumwald 2010; Deikman, Petracek and Heard 2012). Along with yield improvement, efforts need to be taken to improve nutrient content of the existing crops. Nearly 200 million individuals are affected by malnutrition across the globe (FAO 2000). Increased consumption of large quantities of staple crops and a reduced amount of legume seeds in the diet has led to severe micronutrient deficiencies in the human diet. Although these micro-nutrients do not contribute to an individual's energy needs they are essential for growth and healthy development. Malnutrition itself accounts for more than five million childhood deaths across the globe (Bouis and Welch 2010). The focus of agricultural reforms in the past has been to meet energy requirements of the world's poor to avoid famine, whereas the deficiency of micronutrients in the diet inhibits complete utilisation of the energy-giving food and leaves individuals severely malnourished and/or obese. Thus, the focus of agricultural reforms needs to shift towards biofortification of cereal crops. Biofortification can be achieved by traditional agricultural practices like breeding programs, adequate fertilisation and crop rotation (Bouis and Welch 2010). Golden rice presents the success story of fortified cereal with a higher concentration of β-carotene to be converted to deliver retinol (vitamin A) in humans (Tang *et al.* 2009). Achieving biofortification of the micronutrients in crop plants is the first step in the battle with malnutrition, the bioavailability of these nutrients needs to be improved in order to tackle malnutrition. The bioavailability of zinc and iron is severely limited by the presence of substances known as anti-nutrients that interfere with their absorption and utilisation. Phytic acid, the main source of stored phosphorus in grains, acts as an anti-nutrient by chelating to multivalent metal ions like iron and zinc (Kumar *et al.* 2010). Studies have focused on development of low phytate food grains using transgenic approaches to increase bioavailability of other nutrients in the diet (Chen *et al.* 2008b; Amarakoon *et al.* 2012;

Gupta, Gangoliya and Singh 2013; Holm, Kristiansen and Pedersen 2002). Although the low phytate plants have been successful in increasing bioavailability of micro-nutrients, low phytate content in plants can itself reduce basal resistance of the plant to microbial pathogens (Murphy *et al.* 2008). Thus, careful investigation needs to be conducted to evaluate the effect of modified metabolite levels on the general plant responses towards environmental stresses.

1.6. Project overview

This study aimed to explore molecular mechanisms that could play important roles in enhancing rice production and nutritional value for global food security. The first phase of the study aimed to identify key genes involved in the response to multiple stresses in rice that can be exploited in imparting improved yield performance under biotic and abiotic stresses. In drought conditions, simultaneous RKN (*M. graminicola)* infection is known to massively reduce yield, and thus experiments were designed to investigate the response of rice to these two stresses acting in combination. Rice plants (*Oryza sativa* cv Nipponbare) were grown in topsoil in controlled conditions in growth chambers and irrigated to saturation and were subjected to either vegetative drought stress or infected with *M. graminicola* or both simultaneously. Efforts were taken to set up a realistic stress model that could successfully mimic stress conditions encountered by rice plants in the field. Leaf and root tissue samples were collected to perform a microarray based transcriptomic analysis. The Rice Gene chip (Affymetrix) was utilised and microarray analysis was performed at NASC (Nottingham, UK). The transcriptomic changes in the vegetative drought-stressed plants, RKN infected plants and rice plants under simultaneous drought and RKN infection were compared against the changes seen in uninfected, well-watered rice plants, used as controls. Genes responsive to simultaneous drought and nematode stress in leaves and in roots were identified and functionally characterised using reverse genetics.

A field study was performed to validate the stress model used in the microarray study and the results obtained from it. Two lowland rice cultivars, IR64 (indica) and Nipponbare (japonica), were grown during the dry period under upland irrigated conditions at the International Institute of Tropical Agriculture, Nigeria. They were subjected to vegetative drought stress, *M. incognita* infection, simultaneous drought and RKN infection and simultaneous drought and CN, *H. sacchari* infection. The effect of

stress conditions on the growth and yield parameters and hormonal signalling was determined.

The second phase of the study aimed to explore the role of reduced phytate level on nematode susceptibility in plants. To accomplish this aim, low phytate Arabidopsis mutants were subjected to RKN, *M. graminicola* and CN, *H. schachtii* infection in tissue culture. Nematode fecundity and development on the mutant plants was used as a measure of plant nematode susceptibility.

2 CHAPTER

Establishing a glass house model to mimic severe vegetative drought and root-knot nematode, *Meloidogyne graminicola* infection in rice

2.1. Introduction

Plants experience a wide range of environmental perturbances during development that could limit their productivity. These stresses can be classed as (i) abiotic factors such as excessive or insufficient temperature, water or minerals or (ii) biotic factors such as bacterial, fungal or insect attack (Gaspar *et al.* 2002). Environmental stresses are responsible for large scale crop loss each year and with the predicted climate change such losses are expected to increase. For a better understanding of the plant stress responses it is essential to have a model system that can replicate the stresses as they are experienced in the field. The model must be monitored to control the severity of each stress and at the same time be replicable.

2.1.1. The model monocot plant - Rice

Rice (*Oryza spp*) is an important cereal crop that is the staple diet of half the world's population. This figure is set to increase since the demand for rice is predicted to increase by 2,000 million metric tons by 2030 (FAO). 90 % of rice is grown and consumed in Asia-pacific, in flooded lowlands to rain-fed dry land. The major rice producing belts in these regions achieve only 40 % of total production efficiency due to damage caused by drought (Alexander Sarris 2004; Farooq *et al.* 2009). Rice serves as a host for many diseases and pests, 54 in temperate zones and about 500 in tropical countries (Papademetrieu 2009). To improve productivity in any crop it is important to understand the response mechanisms that result in tolerance or susceptibility to a stress. The majority of research on plant stress response pathways has been carried out on the model organism *Arabidopsis thaliana.* However, completion of whole genome sequencing of *Oryza sativa* (rice) has facilitated our understanding of signalling

cascades in rice and other monocot crops, as rice shows 80 % - 90 % synteny with other cereals (Goff *et al.* 2002). Although rice shows only 39 % conservation of gene sequence similarity with the model dicot plant arabidopsis, current research suggests that there are similarities as well as differences in stress signalling pathways between these two species (Salse *et al.* 2002). Therefore, it is important to investigate the responses in this species and other monocot crops. Investigation of stress responsive pathways in rice should lead to the development of technologies to improve future rice and cereal production.

2.1.2. Biotic stress - Root-knot nematode, *M. graminicola*

A total of 200 plant parasitic nematode (PPN) species have been reported to be associated with rice, of which the *Meloidogyne* spp (root-knot nematodes) are most prevalent. *Meloidogyne graminicola,* Golden and Bridge is considered the most serious pest of rice in all ecosystems (Roberts 2007) . It was traditionally reported in the fields of Laos, Vietnam, Burma, Thailand, Philippines, India and the USA. In the last few years, it has been reported to be widespread in rice-wheat growing regions of Nepal, Pakistan and the majority of the upland rice growing regions of India. It can affect lowland rice, upland rice, rice in nurseries and even in deep water production systems (Pokharel *et al.* 2007). The severity of *M. graminicola* infection is dependent on the initial nematode density in soil along with the soil moisture and flooding regimes (Poudyal *et al.* 2005; Soriano, Prot and Matias 2000). Young rice plants grown in drained soil are more susceptible to infection. Juvenile nematodes penetrate the root within five hours after artificial inoculation and many juveniles can enter at the same root tip. The eggs stay viable in moist and water-logged soil for as long as 12 months. The life cycle takes 26-51 days in soil, depending upon the time of the year (MacGowan and Industry 1989). The rice varieties under upland conditions suffer 23.6 % yield loss and susceptible varieties show significant reduction in both fresh and dry biomass, along with reduced grain filling (De Waele *et al.* 2013).

A number of plant parasitic nematode species successfully parasitise the model plant arabidopsis, and its thin, transparent roots and ease to support a whole nematode life-cycle in tissue culture have made it a system of choice for the study of plant-nematode interactions (Sijmons *et al.* 1991). The molecular studies exploring early plant responses and transgenic studies screening plants for PPN resistance have been performed by

artificial inoculation of arabidopsis and seedlings of crop species in tissue culture or in soil-absorbent synthetic polymer like polyethylene glycol (PEG) (Huang *et al.* 2006; Reversat *et al.* 1999; Ingram 1973; Fudali, Wang and Williamson 2012; Teillet *et al.* 2013). In spite of difficulties associated with visualisation of RKN in the thicker roots of crop species, most of the published studies on molecular aspects of *M. graminicola* infection have been performed on rice in tissue culture or in soil-absorbent synthetic polymer (Kyndt *et al.* 2012a; Kyndt *et al.* 2012b; Ji *et al.* 2013a; Ji *et al.* 2013b). The plant responses during the later stage of *M. graminicola* infection and the effect of prolonged infection on plant growth have been studied for rice in glasshouse experiments and in the field (Soriano, Prot and Matias 2000; Padgham *et al.* 2004; Plowright and Bridge 1990; Das *et al.* 2011).

2.1.3. Abiotic stress - water scarcity

Water availability plays a crucial role in rice cultivation. IRRI (1993) has categorized rice land ecosystems into four types depending on the irrigation adopted: irrigated, rainfed lowland, upland and flood-prone rice ecosystems. At least 23 million hectares of rice area i.e. 20 % of the total rice growing area in Asia is affected by drought of different intensities. Drought is the major factor contributing to low and unstable rice production in this area (Pandey 2009). It is a situation where water potential and the turgor of plant leaf cells reduces to a level where normal functions are impaired. It causes stomatal closure and limits gas exchange, reduces water content, turgor, water potential and results in wilting of the plant (Shao *et al.* 2008). Rice is affected by drought stress at each developmental stage and the crop responds differently to the drought stress in different life stages (Boonjung and Fukai 1996). Plants deploy drought avoidance mechanisms including leaf rolling, stomatal closure, reduced tillering and accumulation of osmoprotectants to prevent severe damage caused due to drought (Hadiarto and Tran 2011).

Dehydration by air-drying seedlings grown in hydroponics or tissue culture has been long used as the accepted laboratory method of inducing drought in rice (Claes *et al.* 1990; Dubouzet *et al.* 2003; Rabbani *et al.* 2003). Other commonly used laboratory methods of inducing drought stress include treatment with 400 mM mannitol and PEG to induce osmotic stress, or exogenous application of ABA to mimic the abiotic stress specific hormone response (Lu *et al.* 2009; Rabbani *et al.* 2003; Ouyang *et al.* 2010;

Xiong *et al.* 2010; Yazaki *et al.* 2004). These treatments are characterised by a short span of severe stress in a very early stage of growth that doesn't mimic the stress experienced by crop plants throughout their life span. The laboratory methods have nevertheless been used to evaluate the performance of transgenic plants under severe stress (Bhatnagar-Mathur, Vadez and Sharma 2008). The drought response of a plant is the result of complex interactions between the soil water status and plant water status. Accurate measurement of plant and soil water status is critical for a study that explores the plant responses towards various levels of soil water status. The water status of the soil or the plant can be measured as the water content or the energy status (Jones 2007). The laboratory-based models of drought induction limit the accurate measurement of this complex interaction and thus fail to provide the true degree of stress experienced by the plants. The soil water content is the total free water available in soil that can be taken up by the plant or evaporated by heat. It is widely measured using a straightforward thermogravimetric method, or by using range of soil water content probes (Gardner *et al.* 2000). The water content of a soil, changes with its physical properties such as the particle size (Townend, Reeve and Carter 2002; Cairns *et al.* 2011; Cosby *et al.* 1984). The water content of a plant is the amount of water in the fresh tissue of the plant that is measured as the relative water content (RWC) at a particular point in reference to the water content at saturation (Slayter 1967). The water deficit measurement based on soil and/or plant energy status relies on the state of equilibrium or the gradient in potential energy between the plant tissue and the soil, which determines the uptake of available water from the soil. Tensiometers and dielectric methods are commonly used to measure the soil water potential (Campbell and Gardner 1971; Campbell 1988). Tensiometers measure the soil water potential or the energy a plant will need to extract water from the soil. They comprise of a porous ceramic cup attached with a vacuum gauge placed in close contact with the soil in plant root region. Water moves across the porous cup to equilibrate the soil water potential, this develops a vacuum in the vacuum gauge that corresponds to the readings on the tensiometer (Smajstrla and Harrison 2011). The Dielectric methods use soil moisture probes that give an estimate of soil water content by measuring the soil bulk permittivity. Permittivity is the velocity of an electromagnetic wave or a pulse through the soil. Bulk permittivity of a soil is the result of the relative contribution of the various components such as the air, mineral, organic matter and water. Out of which, water

contributes the most to the bulk permittivity of the soil. The sensor output from the dielectric probe is calibrated empirically to give the volumetric water content of the soil (Muñoz-Carpena, Shukla and Morgan 2004). The leaf water potential is determined by measuring the xylem sap pressure of a plant using a pressure chambers (Boyer 1967). The stomatal conductance or stomatal resistance has been used as an indirect method of measuring plant water status under water deficit (O'Toole and Cruz 1980). Drought stress studies performed in the glasshouse by growing plants in pots or in fields generally define the severity of the treatment by direct or indirect measure of the water status of the plant or the soil (Degenkolbe *et al.* 2009; Jongdee, Fukai and Cooper 2002; Parent *et al.* 2010). They succeed in determining the experimental conditions as a function of the degree of stress experienced by the plants. This increases the specificity of the observed responses towards a particular level of water status of the plant, which is essential for studies that compare plant responses at various degrees of water deficit. An appropriately laid out experimental set up with a defined level of stress increases the reliability and reproducibility of the treatment.

To accomplish the aim of accurately determining transcriptomic changes in rice under vegetative drought stress and *M. graminicola* infection, soil-based pot experiments were first performed in the glasshouse to establish reliable conditions for imposition of each stress. Morpho-physiological parameters were used to indicate the magnitude of damage induced by the two stresses. Methods were optimised for producing replicable and consistent stress responses leading to transcriptomic changes that could be quantified using expression of marker genes. An optimised model, to be used for the microarray analysis, for efficiently inducing drought and nematode stress individually and simultaneously in combination was established. This model replicates the stress regimes encountered by rice plants during vegetative growth phase in aerobic and upland cultivation systems.

2.2. Materials and methods

2.2.1. Rice seed sterilisation

Oryza sativa japonica cv Nipponbare (rice) seeds were surface sterilised by soaking in a solution of 10 % commercial bleach with one drop of Tween-20 in 15 ml solution, for five min. Seeds were then rinsed in water and de-husked using forceps. The de-husked seeds were soaked in 50 % commercial bleach for five min and then washed thoroughly with autoclaved deionised water. The seeds were germinated on sterilised moist filter paper in a Petri dish by incubation in the dark at 20 °C for one week.

2.2.2. Rice growth conditions

After germination, the seedlings were grown in 2 inch cell trays for four weeks in Norfolk top soil (loam) with soluble fertiliser. The cell trays were placed in gravel trays with 2-4 cm of standing water (Figure 2-1 A). After that they were transplanted into 6 inch pots, each pot was placed in an individual saucer and plants were watered from the base through the saucers (Figure 2-1 B). For glasshouse experiments, growth took place at a temperature of 28 °C with 80 % humidity for 11 hours during the daytime and at 22 °C with 80 % humidity for 13 hours during the night. Similar growth conditions were maintained in the Fitotron (Leicestershire, UK) standard growth chamber at light intensity of 350µmol m^{-2} s^{-2}.

2.2.3. Molecular characterisation of *Meloidogyne graminicola*

2.2.3.1. DNA was extracted from single second stage juvenile nematode.

Each nematode was collected in 20 µl water in a 0.5 ml tube. 20 µl NaOH (0.25 M) was added to the tube and centrifuged for 3 min at 6000 g to immerse the nematode in NaOH. At this stage it was either stored at -80 °C or processed directly by incubating overnight at 25 °C in a water bath. The sample was then heated at 99 °C for 3 min. The sample was cooled to room temperature and micro-centrifuged for 1 min at 12,000 g. 4 µl HCl (1 M), 10 µl Tris-HCl (0.5 M, pH 8), 5 µl Triton X-100 (2 %) were added. The mixture was centrifuged at 1100 g for 1 min and then heated to 99 °C for 3 min and again centrifuged for 1 min at 1100 g prior to storage at -20 °C (Floyd *et al.* 2002). A fragment of 18S rDNA was amplified by performing polymerase chain reaction (PCR)

on 2.5 µl of genomic DNA extracted from a single nematode. The PCR was performed using the conditions and reagents described (Table 1-3).

2.2.3.2. Agarose gel electrophoresis

The PCR amplified DNA fragments were analysed by gel electrophoresis. Different strength gels were used depending upon the expected size of DNA fragments.

Gel concentration and expected product

1 % > 1000 bp

2 % 250 bp to 1000 bp

3 % < 250 bp

Depending upon the concentration of gel needed agarose was weighed and mixed in 1 x tris-acetate-EDTA (TAE) (e.g. 1 g agarose in 50 ml TAE for a 2 % gel) and was dissolved by heating in a microwave. The molten agarose was cooled before addition of 1µl of ethidium bromide then swirled to mix well. The agarose was poured into a gel tray with comb and allowed to set. Once set, the tray was placed in a tank that was filled with 1x TAE buffer, the comb was removed and the samples were loaded into the resulting wells. The first and the last well were loaded with a suitable DNA marker, PCR products produced using GoTaq polymerase (Promega) have loading dye in the green buffer thus could be loaded directly. Electrophoresis was carried out at 100V for 30 min, higher concentration gels may require a longer run time. The gel was visualized using a UV trans-illuminator and the image was captured. The desired gel bands were cut using a sterile blade and cleaned by QIA quick gel extraction kit (Qiagen).

2.2.3.3. Ethanol precipitation and ligation

To precipitate the gel extracted PCR amplified DNA fragments , 0.1 volume sodium acetate (3 M, pH 4.8-5.2) and 2 volumes of ethanol were added to the completed PCR reaction and mixed well. The mixture was cooled at -80 °C for 30 min. DNA was pelleted by centrifugation for 10 min at top speed on a table top micro-centrifuge. The ethanol was decanted; 500 µl of 70 % of ethanol was added and spun again for 3 min. The 70 % ethanol was removed to allow the pellet to air dry and the DNA was re-suspended in 3 µl of water. The precipitated PCR products were ligated into 1 µl of pGEMT-easy vector (Promega) using T4 DNA ligase by incubating overnight at 4 °C.

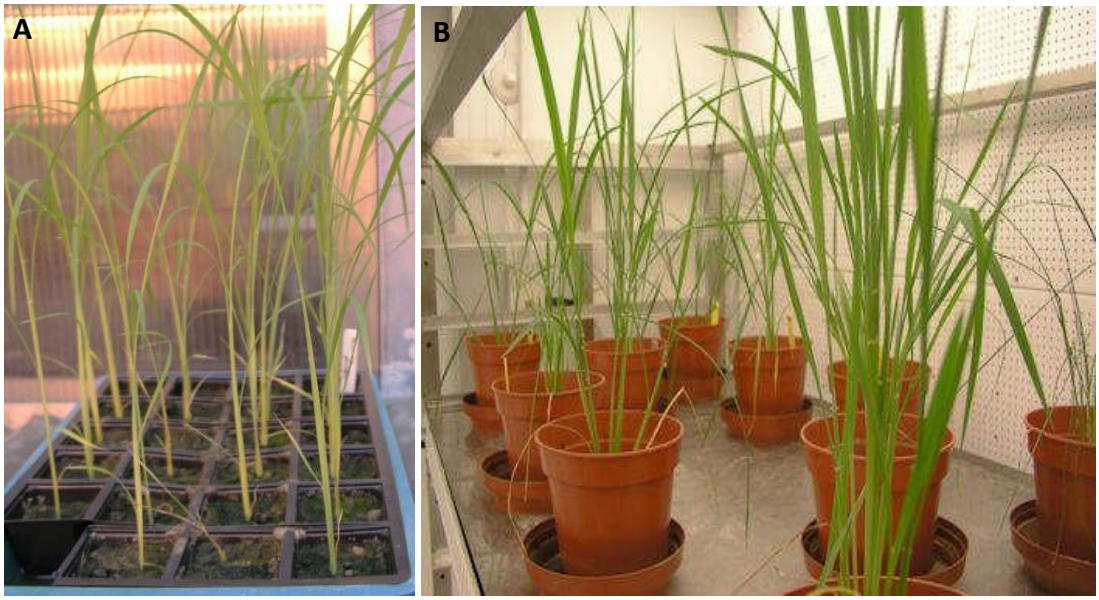

Figure 2-1 Growth conditions for rice plants during the experiment

A. Seedlings in 2 inch cell trays, in a gravel tray with 2-4 cm of water.

B. Four week old (three tiller stage) seedlings transplanted into 6 inch pots, standing in individual saucers with water. The plants were grown in Fitotron standard growth chambers at 28 ºC and 80 % relative humidity with a 12 hour day light period.

2.2.3.4. Transformation

The ligated vector was transformed into competent *Escherichia coli* DH5α cells (Invitrogen). The ligation mixture was added to 100 µl DH5α cells and was mixed well by gently flicking the tube. These cells were incubated on ice for 20 min. The cells were given a heat shock by transferring to 42 °C for 50 s and then again incubating on ice for 2 min. The cells were incubated in a shaking incubator at 37 °C after addition of 500 µl of Luria-Bertani medium (LB .e.i. tryptophan 10 g/ L, yeast extract 5 g/L, NaCl 10 g/L). After one hour these cells were plated onto LB agar plates (LB medium with 15 g/L agar) containing ampicillin (100 µg/ml) and X-gal (40 µg/ml) for blue/white colony selection and incubated overnight at 37°C. The white colonies were picked next day and were inoculated in 5 ml of LB containing 100 µg/ml ampicillin and incubated overnight in a shaking incubator at 37 °C.

2.2.3.5. Restriction digestion

Plasmid DNA was extracted using a miniprep kit (Qiagen) according to the manufacturer's instructions. Restriction digestion was performed on the extracted plasmid DNA in a 20 µl reaction volume. Each reaction mixture comprised of 3 µl of plasmid DNA, 1 µl of restriction enzyme, *Eco*R1 (NEB), 2 µl of enzyme specific reaction buffer (NEBuffer EcoR1) and 14 µl of sterile water. The reaction was incubated at 37 °C for 2-3 hours. The digested DNA was analysed by electrophoresis. Desired gel bands were cut using a sterile blade and difested DNA fragments were gel extracted using the QIA Qiaquick gel extraction kit (Qiagen).

2.2.3.6. Sequencing

The obtained DNA was sequenced using T7 primer at the sequencing facility at University of Dundee, UK. The resulting chromatograms were visualised using Chromas lite 2.01 software and examined by eye. The sequences obtained were searched against all available reference genomic sequences in databases using Basic Local Alignment Search Tool (BLAST) at the National Centre of Biotechnology Information (NCBI). The multiple sequence alignment was obtained using CLUSTALW online tool at the European Bioinformatics Institute (EBI).

Step	Temperature(°C)	Time	Number of cycles
Activation	95	2 min	1
Denaturing	95	1 min	
Annealing	52	1 min 30 sec	35
Extension	72	1 min	
Final extension	72	5 min	1

Table 1 PCR conditions for amplification of 18S rDNA fragment from genomic DNA

Reagent	Volume (µl)	Final concentration in solution
Brilliant III SYBR green master mix	**10**	**2x**
Forward primer	**1**	**400 nM**
Reverse primer	**1**	**400 nM**
Water	**3**	**-**
cDNA	**5**	**As per the experiment**

Table 2 Concentration and volumes of PCR reagents used for amplification of 18S rDNA fragment from genomic DNA

Gene	Forward primer SSU18A	Reverse primer SSU26R	Region amplified
18S rDNA	AAAGATTAAGCCATGCATG	CATTCTTGGCAAATGCTTTCG	~911 bp

Table 3 Primers and size of amplified product from 18S rDNA fragment from genomic DNA

2.2.4. Hatching *Meloidogyne graminicola* juveniles for infection

Meloidogyne stocks were maintained on rice plants on a six week cycle. After six weeks the infected roots were washed to remove the soil and were chopped into 1 cm pieces. Roots were placed on a nylon mesh, on top of a funnel, under a fine mist of water in a mystifying chamber to allow the juveniles to hatch. The flow through was collected in 50 ml tubes and hatched second stage juveniles (J2) that sedimented at the bottom of the tubes were collected every day and were stored at 10 °C in tap water until used.

2.2.5. Infecting rice plants with *Meloidogyne* juveniles

Rice seeds were germinated and grown in the 2 inch cell trays for first four weeks as mentioned in Section 2.2.1 and Section 2.2.2. After that the seedlings were transplanted into 6 inch pots. The pots were half filled with top soil and fertiliser mixture then three 1 cm^2 GF/A filter papers were placed on the soil and 1 ml pipette tips were positioned on to these GF/A filter papers. The seedlings were placed in pots in a way that the root tips touched the GF/A filter papers and were covered with more topsoil and fertiliser mixture. For infecting the plants, *Meloidogyne spp* second stage juveniles were diluted to a known density and a known volume of the nematode suspension was pipetted down the tips. The control plants were mock inoculated by pipetting the same amount of sterilized water through the pipette tips on to the GF/A filter paper. Each pot with an infected plant was placed in an individual saucer containing 2-3 cm of standing water.

2.2.6. The life cycle of *Meloidogyne graminicola* at 28 °C in a glass-house

Two week old seedlings were individually infected with 1000 J2 of *M. graminicola*. Three seedlings were harvested every 24 hours after the inoculation for the next one month. The roots were gently washed to remove soil, soaked in 1 % sodium hypo-chlorite solution for 5 min and rinsed. The roots were placed in boiling 1x acid fuschin (0.035 % - 3.5 g of acid fuschin in 250 ml of acetic acid and 750 ml of water) (Bybd Jr, Kirkpatrick and Barker 1983) for 3 min, rinsed in water and placed in acidified glycerol. The roots in glycerol were incubated at 65 °C overnight for de-staining of the root tissue, and then observed under a stereobinocular microscope (MZ16, Leica). Photographs were taken using the QCapture suite (Qimaging corporation) and the

nematode movement and development in the rice roots was recorded daily to determine the duration of the life cycle of *M. graminicola* at 30 ˚C in a glasshouse in a pot-based set-up.

2.2.7. Extraction of eggs from roots of infected plants

Rice roots infected with *M. graminicola* were chopped into fine pieces and shaken vigorously with 1 % sodium hypochlorite solution for 4 min. The mixture was poured through a set of three sieves (250 µm, 53 µm and 20 µm) stacked in descending order of their mesh size, placed under running water. The chopped roots were washed with running water and eggs were collected from the last sieve (20 µm). Eggs obtained were counted in 1ml aliquots using a Peters counting slide, three replicates counts were performed on each sample.

2.2.8. Relative water content

To determine relative water content of the plant, the fresh weight (FW) of a freshly plucked leaf at different time points in drought treatment was recorded. The leaf was then soaked in water overnight and turgid weight (TW) was noted. The fully saturated leaf was then dried overnight at 60 °C in an incubator and dry weight (DW) was recorded. Relative water content was calculated using the equation -

RWC = (FW-DW) / (TW-DW)

2.2.9. Volumetric soil moisture estimate

The volumetric soil moisture content was estimated using a SM200 moisture sensor probe (Delta-T devices, Cambridge, UK). The 55 mm sensor measures volumetric content of water in 0.5 litre of soil sample. The volumetric soil moisture content is a ratio of the volume of water contained in a sample to the volume of soil in that sample; it is measured in $m^3\ m^{-3}$ and represented as a percentage. A mean of two readings per pot was taken to determine the soil moisture of that pot. The pots with four week old plants were watered to saturation, the initial soil moisture was measured and severity of subsequent drought was calculated as the percentage reduction of this initial soil moisture reading. Because of small differences in the amount of soil in each pot and the

variation in root size the initial reading was different for individual pots, thus the calculations were made individually for each pot.

2.2.10. Gravimetric estimation of evapotranspiration

Alongside the soil moisture content, pot weight was also recorded for each plant. Once the soil moisture content was reduced to the required extent, the evapotranspiration in the previous 24 hours was calculated by taking a difference of two consecutive readings. An equivalent volume of water was supplied to the plant to make up for the loss of water in the previous day. This routine was carried out throughout the drought stress regime or until the samples were collected, as applicable.

2.2.11. Determination of permanent wilting point

To determine the permanent wilting point of the Nipponbare plants, 40 four week old Nipponbare seedlings, after being transplanted into 6 inch pots, were arranged randomly in the glass-house. Thereafter, all plants were watered up to saturation by pouring water into the saucers. After one week watering was withheld from 37 pots whilst three pots were watered in the regular fashion to be used as controls. Volumetric soil moisture was recorded each day (Section 2.2.9). Three plants that showed 10 % reduction in soil moisture content were re-watered on the day one. Three plants showing 20 % reduction in soil moisture content were re-watered on the day two and so on. The rate of survival and recovery were recorded for the plants re-watered at different soil moisture contents. One fully expanded leaf per plant was harvested before re-watering and a second one was harvested 24 hrs after soil saturation and used to calculate the RWC. The RWC and rate of recovery were used to estimate permanent wilting point of cv Nipponbare.

2.2.12. The relationship between RWC and soil moisture content

Four week old rice plants were transplanted into 6 inch pots and watered daily for a week. Thereafter water was withheld from 10 plants whereas three plants were watered daily and used as controls. Soil moisture of all test pots was measured immediately after last watering (0 hr), then subsequently twice each day. Each time the soil moisture was measured, one leaf was harvested from each plant and used to determine the relative water content (RWC). The pot weights were taken at every occasion to also calculate

loss in soil moisture by gravimetric methods. These plants took six days to dry out completely.

2.2.13. Inducing gradual drought stress

To mimic field drought conditions, four week old plants were allowed to dry gradually by withholding water. For the first one week after transplantation into 6" pots seedlings were well watered to allow recovery from transplantation shock. For this period, plants were watered every day by pouring water into the saucers. Volumetric soil moisture content and the pot weight were monitored each day. Watering was withheld for the drought stressed plants until the soil moisture content reached 50 % or 30 % (depending on the experiment requirement) of saturation. A leaf rolling score of 7-8 was also used as the visible measure of drought stress. Thereafter, the plants were supplied daily with the volume of water lost due to evapotranspiration (determined gravimetrically) during that day.

2.2.14. Effect of initial inoculum density of *M. graminicola* on rice

After transplanted into 6 inch pots, the four week old plants were watered and allowed to recover from transplantation shock. A week later, the plants were divided into four groups of six plants each. Group one was left uninfected and maintained as control. All plants in remaining three groups were infected with 1000, 3000 or 9000 *M. graminicola* J2's each as described in Section 2.2.5. Two fully expanded leaves other than the oldest leaf were collected, one was collected two days post infection and another one was collected four days post infection and snap frozen in liquid nitrogen for expression analysis of stress responsive genes. All the plants were harvest 40 days post infection (approximately after two generations of *M. graminicola*). At harvest, morphological and yield parameters were recorded along with the number eggs obtained after two generations of *M. graminicola* infection.

2.2.15. Optimisation of simultaneous drought and nematode stress

Eighteen plants were transplanted in 6 inch pots and allowed to recover for a week. A week later, six plants were gradually dried and maintained at 30 % soil moisture whereas another set of six plants was gradually dried and maintained at 50 %, as described in Section 2.2.13. The remaining five plants were watered every day and

maintained at 100 % soil moisture content. After ten days of drought stress, all three sets of plants were infected with 3000 J2s as described in Section 2.2.5. The drought stressed plants were maintained at 30 % and 50 % soil moisture content and the remaining six plants were well watered. Four days post infection; three plants from each set were harvest. A fully expanded leaf other than the oldest leaf was collected from each plant and snap frozen using liquid nitrogen for expression analysis of stress responsive genes. The roots were rinsed in water to remove soil and 1-2 cm pieces of root tips were also collected and snap frozen for expression analysis of stress responsive genes. All plants were harvested 40 days post infection and the number of eggs produced per plant was determined.

2.2.16. Expression analysis of stress responsive genes

2.2.16.1. RNA extraction

The frozen plant material was ground to a fine powder using liquid nitrogen. The total RNA was extracted using a RNeasy plant minikit (Qiagen) according to the manufacturer's instructions. 100 mg of the tissue sample was mixed with 450 µl of buffer RLT (pre-mixed with 10 µl/ml of β-mercaptoethanol) applied to a QIAshredder column and centrifuged for 2 min at full speed. The supernatant was transferred to a fresh tube, diluted with half the volume of absolute ethanol and mixed well by inverting. This solution was then transferred into a RNeasy column and microcentrifuge for another 15 sec at 10,000 rpm. The flow through was discarded and the column was washed using 350 µl of buffer RW1 and microcentrifuged again for 15 sec at 10,000 rpm. To remove traces of DNA, an on-column DNase digestion was performed using the RNase Free DNase set (Qiagen, cat. no. 79254). To perform the digest, 10 µl of DNase was mixed with 70 µl of RDD buffer; the mixture was applied onto the column, and incubated at room temperature for 15-20 min. The column was then washed with 350 µl of buffer RW1, microcentrifuged at 10,000 rpm for 15 sec. The flow through was discarded and the column was washed with 500 µl of buffer RPE to remove contaminants. The total RNA was eluted in 50 µl of sterile water and stored at -80 °C. The concentration and purity of the RNA was determined using a Nanodrop ND-1000 spectrometer (Thermo Scientific). A 1 µl sample of RNA was analysed at a wavelength of 260 nm, 260/280 nm and 260/230 nm ratios were recorded.

2.2.16.2. cDNA synthesis

The quantified RNA was subjected to reverse transcription to prepare cDNA. Equal quantity of RNA obtained from each biological replicate in the experiment was pooled to give 1 µg of total RNA in each cDNA reaction mixture of 20 µl. This was mixed with 1µl of an anchored oligo dT primer stock (10 µM) and 1 µl of dNTP stock (10 mM each dNTP) 1 The reaction mixture was heated to 65 °C for 5 min and then cooled on ice. Then 4 µl of 5x reaction buffer, 2 µl of DTT (0.1 M) and 1 µl of RNase-free water was added. This reaction mixture was then incubated at 42 °C for 2 min. Following incubation, 1 µl of Superscript II reverse transcriptase (Invitrogen) was added to the reaction mixture and it was heated at 42 °C for 50 min. Incubation at 70 °C for 15 min inactivated the enzyme prior to storage at -20 °C.

2.2.16.3. Real- time quantitative PCR

Real-time quantitative PCR (qPCR) was performed using cDNA prepared from total RNA extracted from the leaves of control and stressed plants. The tumour homologue protein (*tph*) gene was used as the normaliser because of its stable expression profile in tissues at all stages of development under various stress conditions (Narsai *et al.* 2010). The mRNA sequences for the stress responsive marker genes were obtained from the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/index.shtml) (RGAP). The primers for qPCR were designed using the latest version of Primer3plus web interface (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) with standard qPCR settings; Tm= 60 °C ± 1 °C, GC content 50 – 60 %, primer length ~ 20 nucleotides, and product length of 100-150 bp, with primers spanning an intron. The primers were synthesised and obtained from Sigma Aldrich. The qPCR was performed using the reagents and the cycling programme detailed in Tables 4 to Table 6. Ten-fold or five-fold dilution series of this cDNA, depending on basal expression of the genes , were used to determine the efficiency and specificity of each primer pair. Primers with an efficiency of 100 % ± 5 % with an R^2 value of nearly 0.98, amplifying a single product were chosen and used for each gene. The cDNA obtained from a 20 µl cDNA reaction was diluted five-fold and 5 µl of this dilution was used with optimised primers for expression analysis in the stressed tissue. Triplicate technical replicates were performed for each reaction. Figure 2-2 and 2-3 show representative standard curve, dissociation curve and amplification plot in control and all three treatments for the normalising gene

TPH, which was used to normalise the expression of the target genes across the treatments.

2.2.17. Imposition of simultaneous drought and nematode stress

Forty plants were transplanted in 6 inch pots and allowed to recover. A week later they were divided into four groups, group A – control plants (well watered - uninfected), group B – drought stressed plants (maintained at 50 % soil moisture and un-infected), group C – nematode stressed plants (well-watered and infected with 3000 J2s each) and group D – multiple stresses (maintained at 50 % soil moisture and infected with 3000 J2s each). Pots were labelled according to the treatments and randomly arranged in a controlled growth chamber. The plants in group B and D were gradually dried to 50 % soil moisture content and there after maintained at the same. The plants in group A and C were well watered throughout the experiment. After the plants in group B and D had suffered drought stress for ten days, the plants in group C and D were infected with 3000 J2s each and the plants in group A and B were mock inoculated with same amount of sterilised water. All plants were harvested forty days post inoculation. At harvest shoot fresh weight, root fresh weight, height of plant, tiller number, number of panicles and grain weight per plant were recorded. The whole shoot system was dried in a 60 °C incubator overnight and dry weight was recorded.

Step	Temperature (°C)	Time	Number of cycles
Denaturing	95	10 min	1
Segment 1	95	30 sec	40
Amplification	60	1 min	
Segment 2	95	1 min	1
Dissociation curve	60	30 sec	
	95	30 sec	

Table 4 Real-time quantitative PCR conditions for expression analysis of stress responsive genes

Reagent	Volume (µl)	Concentration
Brilliant III SYBR green master mix	10	2x
Forward primer	1	500 nM
Reverse primer	1	500 nM
Water	3	-
cDNA	5	As per the experiment

Table 5 Real-time quantitative PCR reaction mix for expression analysis of stress responsive genes

Gene locus	Forward primer	Reverse primer
OsDREB2B1	TCCAGCCCGGAAGAAAATGT	GCTCCTGCTGATTGTTGAGC
OsDREB2B2	CAGCCCGGAAGAAAAAGCG	GCTCCTGCTGATTGTTGAGC
OSDREB2A	GCTGCACATCAGCACCTTCA	TCCTGCACCTCAGGGACTAC
OsPR1a	GTCGGAGAAGCAGTGGTACG	GGCGAGTAGTTGCAGGTGAT
OsPR1b	GCCATGGCACTCCCCTCCCA	CTGCACGCTCGTGTCCCAGG
OsPR1c	ACGAGCCCCGTGGCAACATC	GCGCGTGCATGTAACGAGCG
TPH(normaliser)	CATTGGTGCCAACCCATC	AAGGAGGTTGCTCCTGAAGA

Table 6 List of primers for stress responsive genes to be used as markers of biotic and abiotic stress

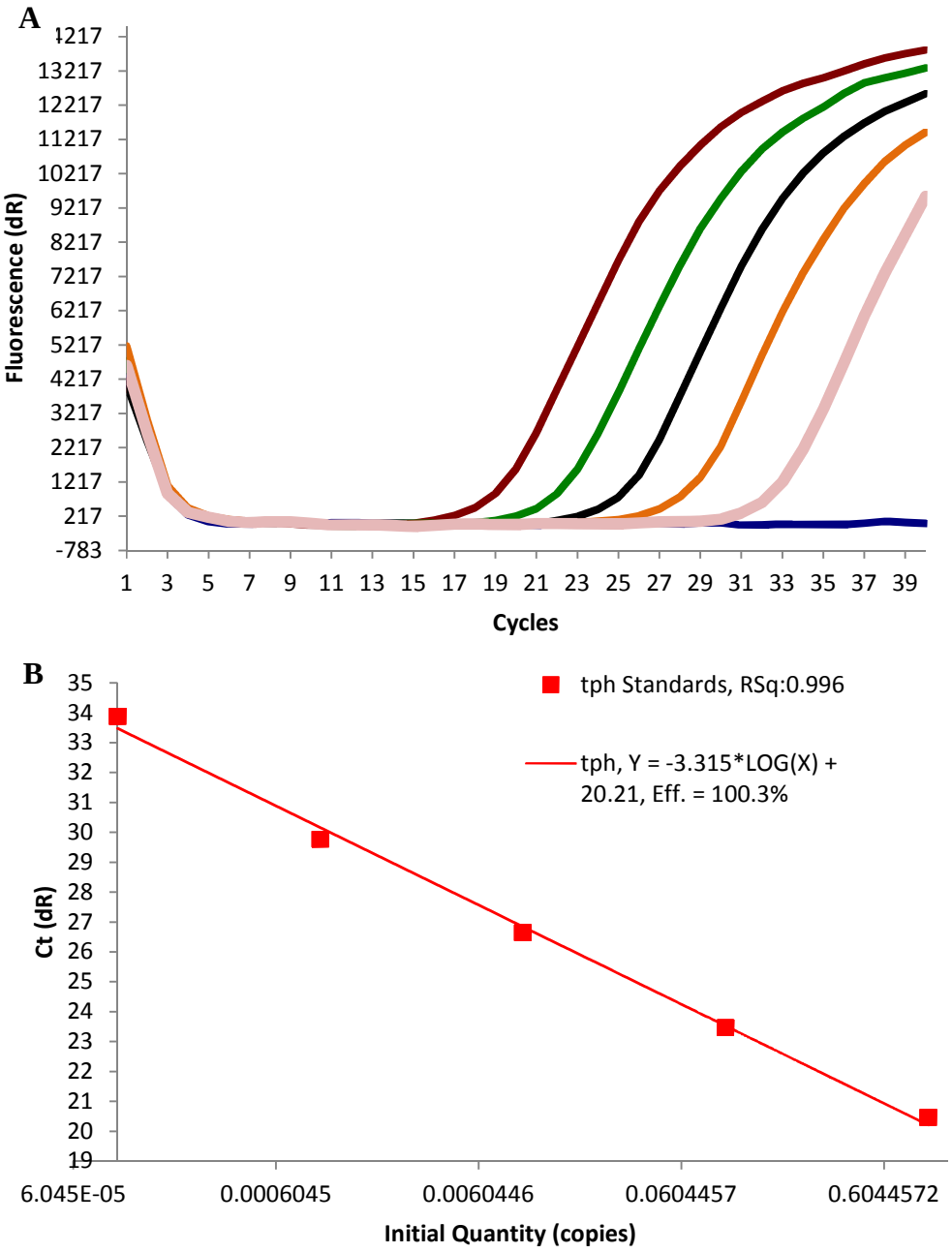

Figure 2-2 Standard curves and amplification plots for the primer pair used to amplify normalising gene tumour homologue protein (*TPH*).

The first panel shows the amplification plot for the *TPH* transcripts amplified using a primer concentration of 500 nm with -10 X dilution series of the template cDNA. The second panel shows a standard curve with a R^2 value of 0.996 and calculated efficiency of the primer pair used (100.3 %).

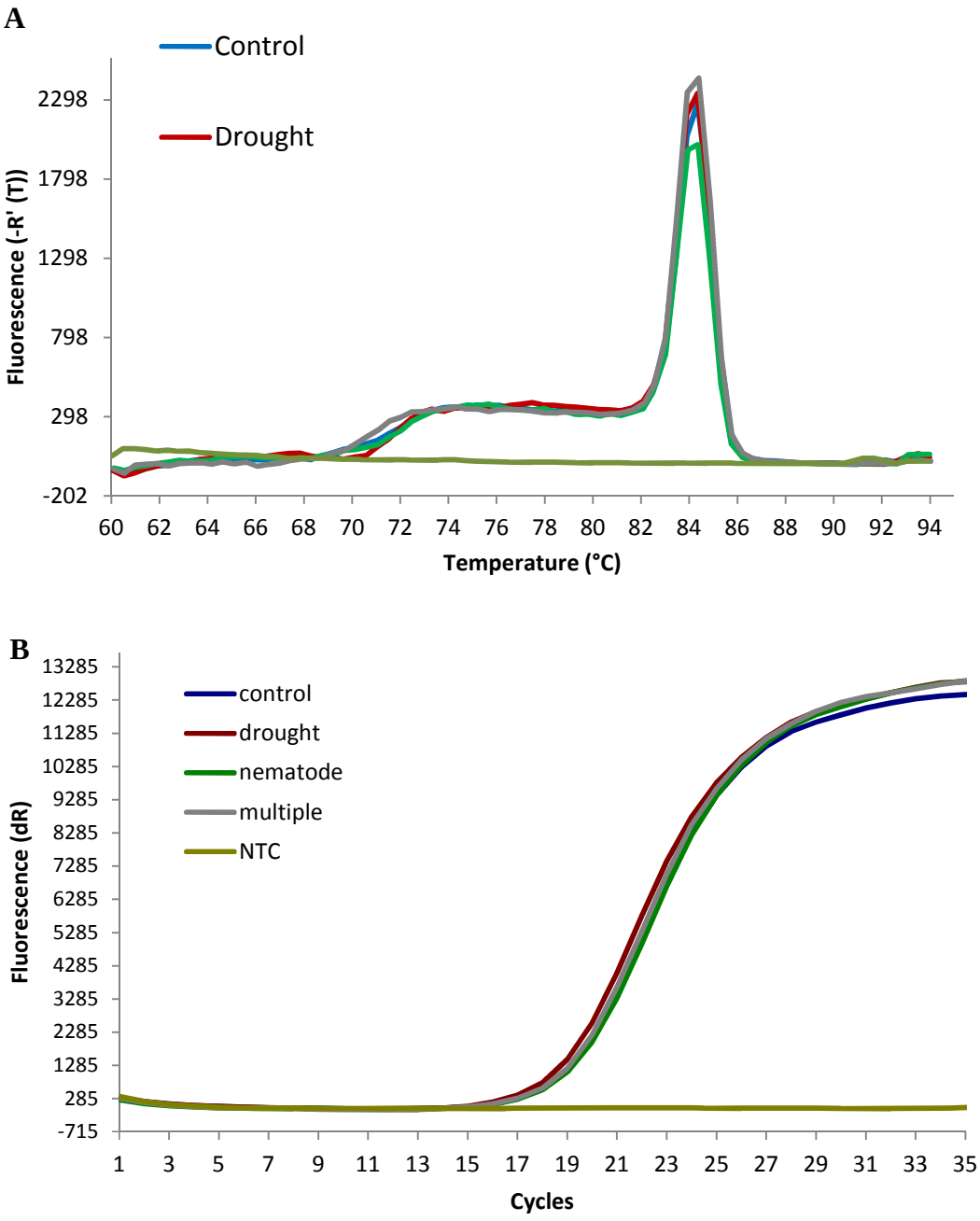

Figure 2-3 The dissociation curve and amplification plots for the primer pair used to amplify the normalising gene tumour homolog protein (*TPH*) in the treatment samples

The transcripts were amplified using a primer concentration of 500 nm each with -5 times diluted template cDNA synthesized from a 1 µg RNA. The dissociation curves show specificity of the primer pair. The second panel shows the amplification plot for the same. The relative starting quantity of cDNA was determined by recording the number of cycles needed the sample to reach the threshold fluorescence compare to the normalising gene. The relative quantity of a transcript in samples from different stress treatments was determined by comparing to the expression in the un-stressed plants (control) relative to the expression of normalising gene in corresponding stress treatment.

2.3. Results

2.3.1. Molecular characterisation of *M. graminicola* isolates

For molecular characterisation of established *M. graminicola* colonies, a 900bp fragment of 18S rDNA was PCR amplified from genomic DNA extraction carried out on single second stage juveniles (Figure 2-4 A). The amplified fragments from three second stage juveniles were cloned (Figure 2-4 B). The sequences obtained were compared against those in databases (Figure 2-5). The 18S rDNA sequences obtained for three individuals were identical to each other. These sequences were similar to the 18Smeloidogyne05 alignment group (includes *M. graminicola*, *M. nasi*, *M. minor*, *M fallax* and *M. chitwoodi*) on the Plant-Parasitic Nematodes Multilialigned Ribosomal Cistrons database (PPNEMA). The sequences are 99.5 % similar to the *M. graminicola* sequence, accession no - AF442196.1 (Figure 2-5). The characteristic hooked galling in rice, further confirmed that the colony obtained was *M. graminicola*.

2.3.2. *M. graminicola* life cycle at 28 °C

M. graminicola shows a temperature dependent life-cycle at different times of the year. The life-cycle of *M. graminicola* was studies at 28 °C and 80 % humidity on rice cv Nipponbare under controlled environment. The two-week old plants were inoculated by pipetting J2 nematodes onto the root tips. The second stage juveniles of RKN are known to enter the plant behind the root tip and migrate towards the root tip, take a U-turn and enter the vascular bundle travelling intercellularly. They establish a feeding site in the vascular tissue and the neighbouring pericycle cells start dividing to form a gall (Dutta, Ganguly and Gaur 2012; Luc, Sikora and Bridge 2005). In the glasshouse experiment, on rice plants grown at 28 ºC in soil, the second stage juveniles were seen entering the roots 24 h after inoculation. The root tips start showing swelling on day two. On day four, maximum numbers of juveniles that have entered the roots were seen to have established a feeding site in the vascular bundle. By nine days post inoculation (dpi), visible galls were seen at the point of infection. The root tissue around the infection point started to become denser and thick. At 12 dpi maximum number of third stage juveniles were observed in the roots. At 15 dpi the fourth stage juveniles that can be distinguished into males and females could be seen. Eighteen dpi mature females started to lay eggs. Twenty days after inoculation, the roots could be used to perform egg extractions to estimate the level of infection or to obtain juveniles using the mistifier

technique. The life cycle of *M. graminicola* as observed at 28 °C and 80 % humidity is summarised in the Figure 2-6.

2.3.3. Initial inoculum density of *M. graminicola* on rice

To determine the number of juveniles required to initiate an optimum infection level and systemic defence response, plants were infected with 1000, 3000 or 9000 juveniles (six plants each). The nematodes were sustained for two generations, i.e. forty days before the plants were harvested. Infection with 9000 nematodes significantly stunted the plants as compared to the uninfected plants. It also significantly reduced plant biomass and root size. The infection level did not have a significant effect on the number of tillers or number and weight of inflorescences produced by the plant (Figure 2-7).

2.3.4. Expression of pathogenesis related genes

The expression analysis of pathogenesis related genes, to be used as markers of biotic stress in further experiments was performed using qPCR. The relative expression levels of genes PR1a, PR1b and PR1c in plants infected with 1000, 3000 and 9000 *M. graminicola* compared to uninfected control plants were studied. The expression analysis was performed on the youngest (y), the oldest (o) and a mature leaf apart from the oldest leaf from each plant in each infection regime. The expression was analysed at two different time points, 2 dpi and 4 dpi. The PR1b gene in mature leaf showed induction in expression proportional to the number of nematodes used to infect at 4 dpi and an insignificant change in expression at 2 dpi (Figure 2-8). An initial inoculum density of 3000 juveniles per plant was chosen for further experiments as it was sufficient to induce a detectable systemic response in plants and did not reduce the plant growth. Thus a fully expanded leaf other than the oldest leaf was sampled for transcriptomic analysis.

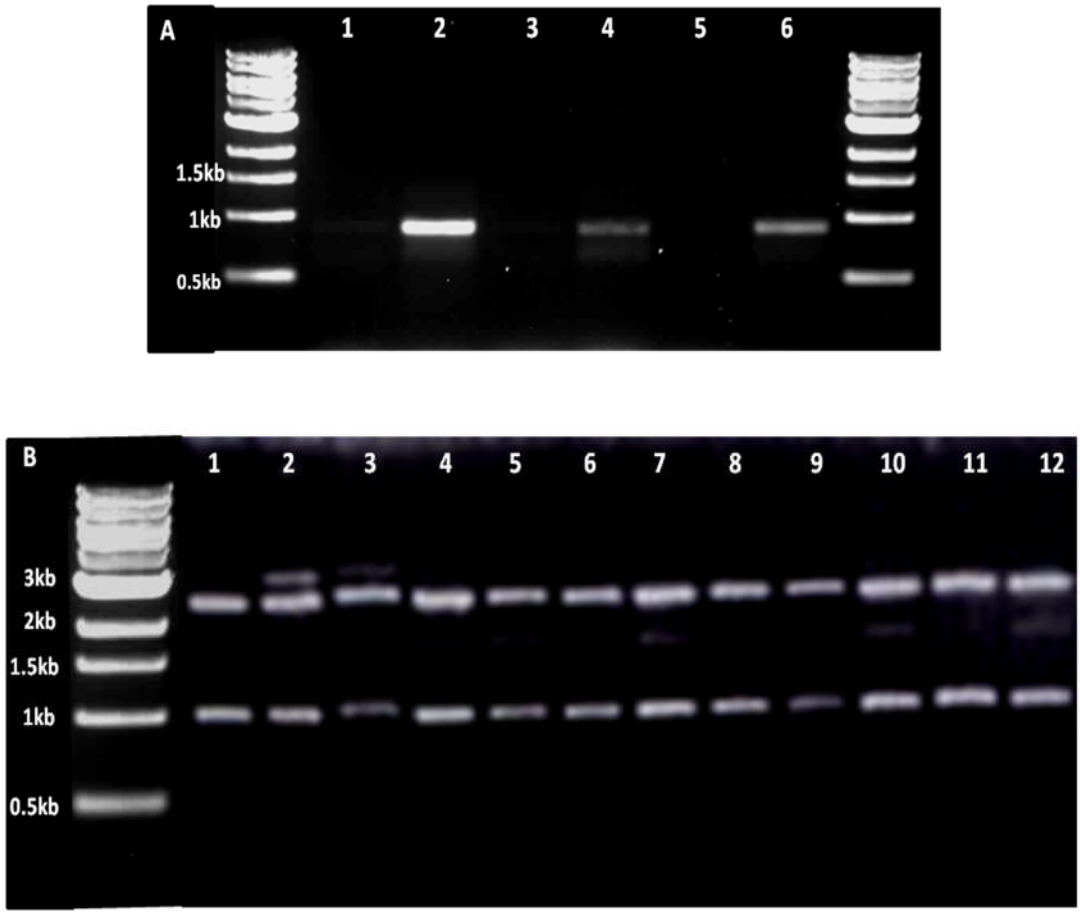

Figure 2-4 The 18S rDNA fragment amplified from the genomic DNA of single nematodes of *M. graminicola* colony.

A. PCR products amplified from single nematode DNA extract using the SSU18A and SSU26R primer pair. Lanes 1-6 show the product from *M. graminicola*, run along with the 1kb molecular marker. These fragments were cloned into pGEMT easy vector and transformed in *E. coli* cells.

B. The restricted products obtained from the digestion of ligated plasmid from various transformed bacterial colonies. The samples that have undergone complete digestion show two bands, the smaller fragment of size 911 bp corresponds to the amplified region of 18SrDNA. The bigger fragment is the vector back bone.

```
M. graminicolaAACGGCTCATTACAATGGCCATGATTTACTTGATCTTGATTAATCCTAAATGGATAACTG
  AF442196.1  AACGGCTCATTACAATGGCCATGATTTACTTGATCTTGATTAATCCTAAATGGATAACTG

M. graminicolaTGGAAAATCTAGAGCTAATACATGCACTAAAGCTTTGACCTTACGGAAAAGCGCATTTAT
  AF442196.1  TGGAAAATCTAGAGCTAATACATGCACTAAAGCTTTGACCTTACGGAAAAGCGCATTTAT

M. graminicolaTAGAACAAAACCACGCGGCTTCGGCTGCTTTTTGTTGACTCAGAATAACTAAGCTGACCG
  AF442196.1  TAGAACAAAACCACGCGGCTTCGGCTGCTTTTTGTTGACTCAGAATAACTAAGCTGACCG

M. graminicolaCATGGCCTCTGTGCCGGCGGCGTGTCTTTCAAGCGTCCACTTTATCAACTTGACGGGAGC
  AF442196.1  CATGGCCTC-GTGCCGGCGGCGTGTCTTTCAAGCGTCCACTTTATCAACTTGACGGGAGC

M. graminicolaATAATCGACTCCCGTGGTTGTGACGGATAACGGAGGATAAGGGTTCGACTCCGGAAAAGG
  AF442196.1  ATAATCGACTCCCGTGGTTGTGACGGATAACGGAGGATAAGGGTTCGACTCCGGAGAAGG

M. graminicolaGGCCTGAGAAATGGCCACTACGTCTAAGGATGGCAGCAGGCGCGCAAATTACCCACTCTC
  AF442196.1  GGCCTGAGAAATGGCCACTACGTCTAAGGATGGCAGCAGGCGCGCAAATTACCCACTCTC

M. graminicolaGGCTCGAGGAGGTAGTGACGAGAAATAACGAGACCGTTCTCATTGAGGCCGGTCATCGGA
  AF442196.1  GGCTCGAGGAGGTAGTGACGAGAAATAACGAGACCGTTCTCATTGAGGCCGGTCATCGGA

M. graminicolaATGGGTACAATTTAAACCCTTTAACGAGTATCAAGGAGAGGGCAAGTCTGGTGCCAGCAG
  AF442196.1  ATGGGTACAATTTAAACCCTTTAACGAGTATCAAGGAGAGGGCAAGTCTGGTGCCAGCAG

M. graminicolaCCGCGGTAATTCCAGCTTCTCCTAATGCATAGAATTATTGCTGCGGTTAAAAAGCTCGTA
  AF442196.1  CCGCGGTAATTCCAGCTT-TCCTAATGCATAGAATTATTGCTGCGGTTAAAAAGCTCGTA

M. graminicolaGTTGGATTCGTATCGATACTCTGGAACCCTTCGGGTGTTTCTGGATGTTATCGATTTTTAT
  AF442196.1  GTTGGATTCGTATCGATACTCTGGAACCCTTCGGGTGTTTCTGGATGTTATCG-TTTTAT

M. graminicolaCGTAATGTTCGGTTTTGAGTCCTTAACAGGATTCTTAACAGGCATTGCAAGTTTACTTTG
  AF442196.1  CGTAATGTTCGGTTTTGAGTCCTTAACAGGATTCTTAACAGGCATTGCAAGTTTACTTTG

M. graminicolaAACAAATCAGAGTGCTTCAAACAGGCGTATTCGCTTGAATGATCGTGCATGGAATAATAG
  AF442196.1  AACAAATCAGAGTGCTTCAAACAGGCGT-TTCGCTTGAATGATCGTGCATGGAATAATAG

M. graminicolaAAAACGATTTCGGTTCAGTTTTATTGGTTTTACGGACTGAGATAATGGTTAACAGAGACA
  AF442196.1  AAAACGATTTCGGTTCAGTTTTATTGGTTTTACGGACTGAGATAATGGTTAACAGAGACA

M. graminicolaAACGGGGGCATTTGTATGGCCACGTGAGAGGTGAAATTCTTGGACCGTGGCCAGACAAAC
  AF442196.1  AACGGGGGCATTTGTATGGCCACGTGAGAGGTGAAATTCTTGGACCGTGGCCAGACAAAC

M. graminicolaTACAGCGAAAGCATTTGCCAAGAATGAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCG
  AF442196.1  TACAGCGAAAGCATTTGCCAAGAATG--------------------------------
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Figure 2-5. Sequence identity of the 18S rDNA sequence amplified from a single *M. graminicola* juvenile with the 18S rDNA sequence of *M. graminicola* (GenBank:AF442196.1) using ClustalW.

The single nucleotide polymorphisms are highlighted.

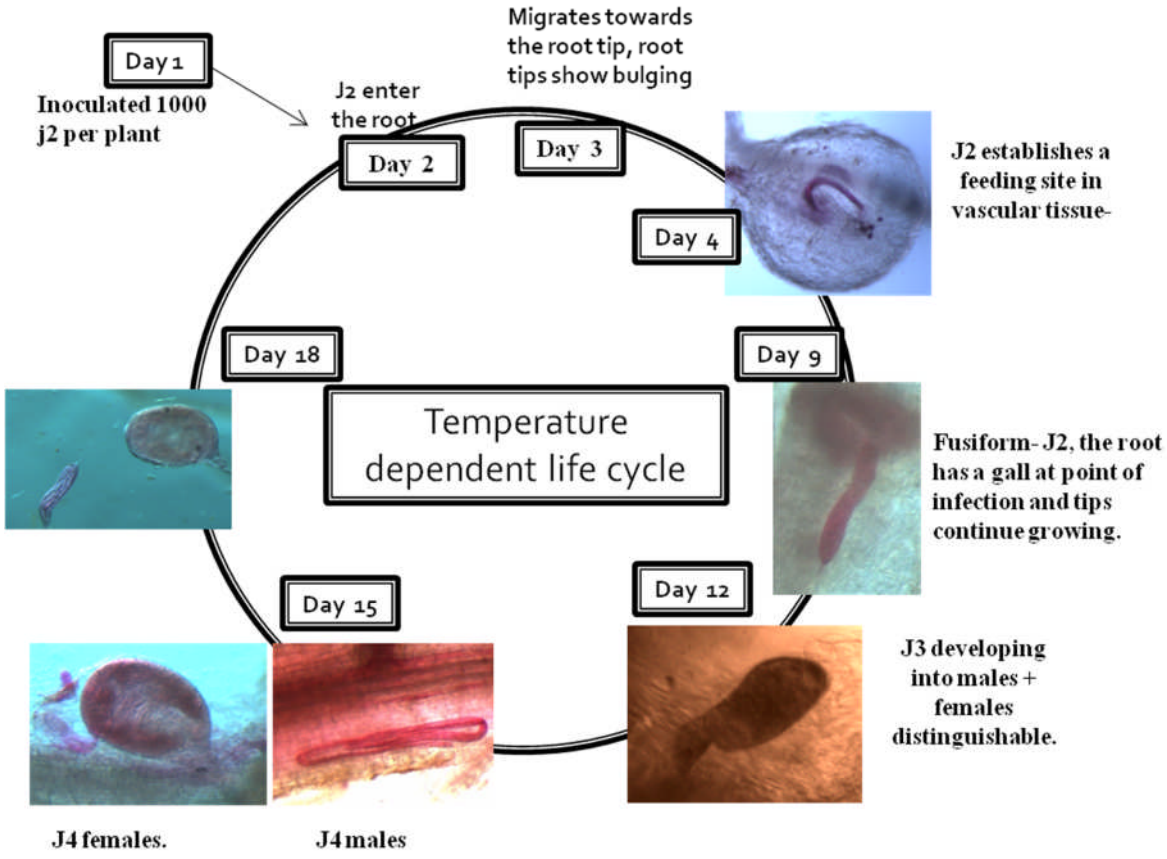


Figure 2-6 Life cycle of *M. graminicola* at 28 ºC on rice cv Nipponbare in soil.

M. graminicola follows a 20 day life-cycle from the point at which the juveniles enter the root until they become gravid on the rice cv Nipponbare at 28 ºC in soil. Two days after inoculation juveniles are seen entering the roots, they travel intercellularly towards the root tip, take a U-turn and infect the vascular bundle by day four, establishing a feeding site. A visible gall appears at the site of infection by day nine post inoculation. Third stage juveniles were observed 12 dpi. By 15 dpi, the fourth juvenile stage males and females are distinguishable. At 18 dpi, adult gravid females are observed. At 20 dpi, mature root galls can be used for egg extractions or obtaining second-stage juveniles using a mistifier technique.

2.3.5. Permanent wilting point of Nipponbare

The permanent wilting point of Nipponbare was determined to explore drying pattern of Nipponbare plants for soil based pot study. The rate of recovery was accessed by re-watering the plants at different soil moisture content. The plants that were re-watered at soil moisture level below 20 % or had watering withheld for seven days failed to recover. This established a baseline for the further drought stress experiments.

2.3.6. Relative water content of plants and soil moisture content

To non-destructively estimate plant water content, a relationship was established between the RWC of leaves and the soil moisture. With progression of severe drought the soil moisture and gravimetric pot weight reduced significantly in stressed plants (test) as compared to the well-watered control plants ($p < 0.05$) (Figure 2-9 A - B). There was no major difference between the RWC of leaves of control plants and stressed plants for the initial 54 hours of drought but thereafter a gradual significant drop in RWC of leaves of stressed plants was observed. Figure 2-10 shows the variation in RWC of leaves of stressed and the control plants during the course of drought stress. Figure 2-11 A and 2-11 B shows the relation between volumetric soil moisture and gravimetric loss in pot weight with the RWC of the leaves calculated in due course of drought stress, respectively. The data show strong correlation between the soil moisture and loss of moisture from the pot with RWC of leaves. The volumetric soil moisture content and gravimetric loss in pot weight decreased proportionally to the RWC of leaves of the drought stressed plants. The leaf rolling score was used as the visible marker to assess severity of drought stress in the plants

2.3.7. Optimisation of simultaneous drought and nematode stress treatment

To induce simultaneous drought and nematode stress on plants, the optimum soil moisture level favourable for *M. graminicola* infection was determined. Plants under stress regimes were arranged randomly in the growth chamber (Figure 2-12 A). The soil moisture of pots was monitored and maintained at 30 % or 50 % of saturation level by using volumetric soil moisture content and gravimetric measurements as the non-destructive measures of water status of the plant. The plants were simultaneously

infected with 3000 juveniles of *M.graminicola* (Section 2.2.15). Hooked galling of root tips (Figure 2-12 B) was used as the non-destructive indicator of successful nematode infection and leaf rolling score (Figure 2-12 C-D) was used as the indicator of the progressive drought stress. Up to 70 % reduction in volumetric soil moisture accompanied by infection with 3000 RKN juveniles significantly reduced the root mass (Figure 2-13). Thus it was decided to maintain the plants at 50 % soil moisture level for the simultaneous drought and nematode infection experiments

2.3.8. Simultaneous drought and nematode stress model

As established by above discussed experiments plants were maintained at 50 % soil moisture and infected with 3000 juveniles for successful detectable simultaneous stress responses. As leaves respond immediately to a change in water status of the soil, expression analysis of drought responsive genes was performed on root tissue to see if the drought stress induced was severe enough to modulate changes throughout the plant or just in leaves. The expression of drought responsive genes *DREB* genes in root tissue confirmed the induction of uniform drought stress. The two splice variants of gene *DREB2B*, *DREB2B1* and *DREB2B2* showed significant six fold up-regulation in drought stressed and seven fold up-regulation in simultaneous multiple stressed plants. Both of these transcripts were also up-regulated in nematode stressed plants but at a lesser magnitude (Figure 2-14A). The systemically induced response to nematode attack was studied by expression analysis of pathogenesis related genes in leaves at 4 dpi. All three pathogenesis related genes *PR1a*, *PR1b* and *PR1c* were significantly up-regulated in all three treatments. The highest relative fold change was observed in expression of *PR1a* gene in multiple stressed plants. In nematode stressed samples, the expression of *PR1c* was higher than that of the other two genes (Figure 2-14B). The plants under the three stress regimes were harvested 40 days post-inoculation and phenotypic characteristics of plant height, tiller number, number of inflorescences, root fresh weight and shoot fresh and dry weight were recorded (Figure 2-15 and 2-16). The plants that endured severe drought and simultaneous drought and nematode stress for 40 days were significantly more stunted than the control and nematode infected plants ($p \leq 0.005$). The nematode infected plants had a greater number of tillers than the other two treatments and controls ($p \leq 0.05$). The drought and simultaneously multiple stressed plants produced very few inflorescences ($p \leq 0.0001$) Drought had a significant negative

effect on the root mass, but the effect was less severe when the plants were drought stressed in conjugation with nematode infection (drought $p \leq 0.005$ and multiple $p \leq 0.01$). A similar effect was seen on the fresh weight of shoots. The nematode infected plants had higher fresh shoot mass than the control plants ($p \leq 0.05$). Although, there was no significant difference in the dry shoot weight of control and nematode stressed plants, the drought and multiple stressed plants experienced the same magnitude of reduction in the weight ($p \leq 0.001$)(Figure 2-16 A). Significantly fewer eggs were produced after two generations of *M. graminicola* infection when this was combined with drought stress ($p \leq 0.01$)(Figure 2-16 B). The drought and multiple stressed plants also had significantly reduced yield per plant; the mean grain weight per plant was significantly reduced when compared to the unstressed plants ($p \leq 0.005$) (Figure 2-16 C).

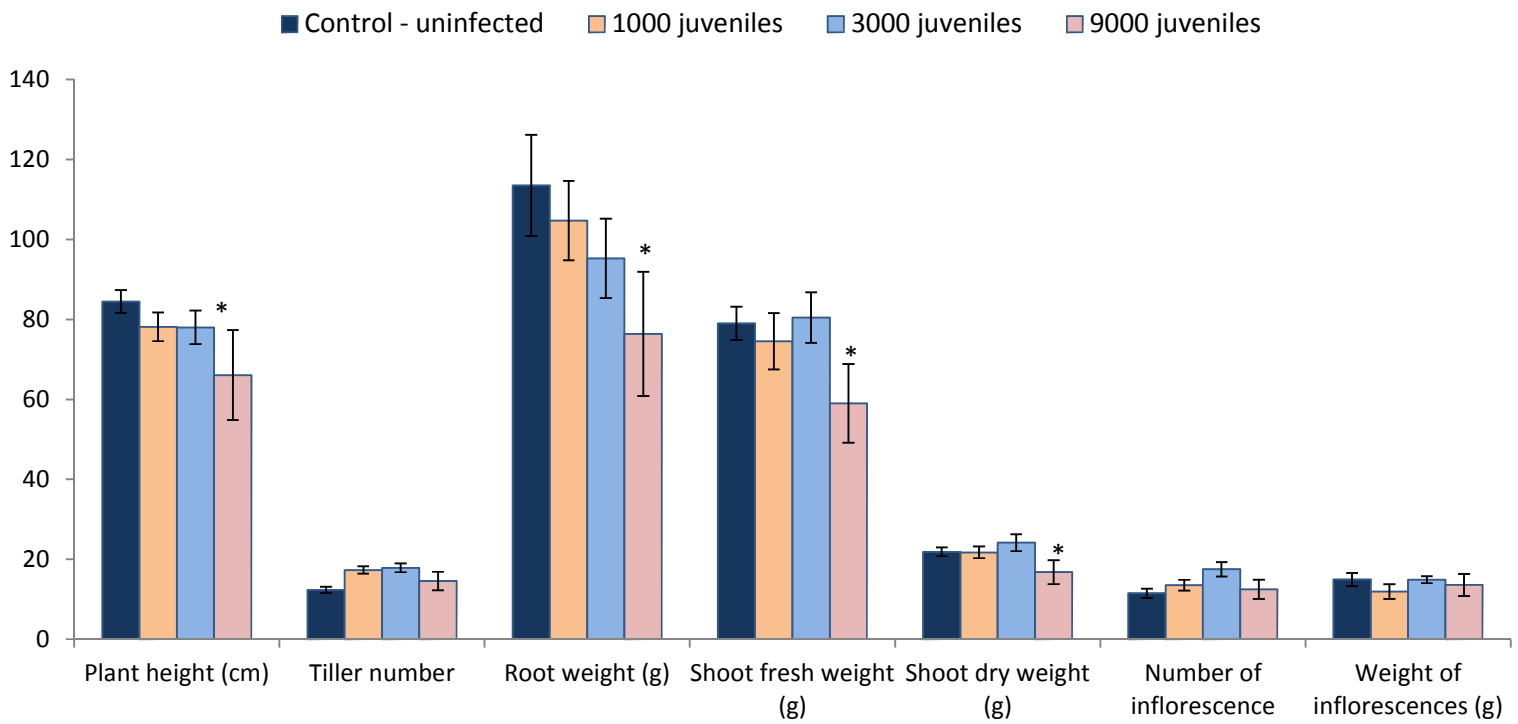


Figure 2-7 Phenotypic characterisation of rice plants under different regimes of *M. graminicola* infection.

Four week old plants were infected with 0 (control), 1000, 3000 and 9000 second stage juveniles. The plants were harvested after two generations of RKN infection i.e. forty days post inoculation. Plant height, tiller number, root and shoot fresh weight, shoot dry weight, number and weight of inflorescences produced were recorded. Each bar denotes the mean of five plants under each infection regime, error bars show standard error of the mean. The significant difference of each regime from the control was determined using the Mann-Whitney U test. * = $p \leq 0.05$.

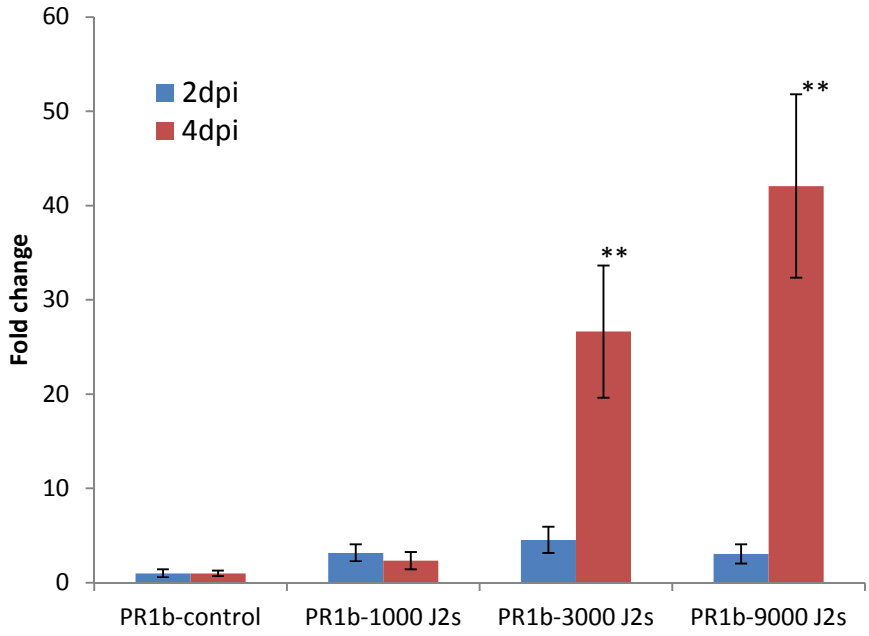


Figure 2-8 Expression analysis of pathogenesis related gene (*PR1b*) in a mature leaf of the rice plants under *Meloidogyne graminicola* infection at 2 dpi and 4 dpi.

The systemic induction of pathogenesis related gene *PR1b* was analysed using real time quantitative PCR in a mature leaf apart from oldest (m) at two different time points (2 dpi and 4 dpi). Expression under each infection regime relative to the expression in control plants was represented as the mean of three technical replicates, with the error bars showing standard error of the mean. Each technical replicate had pooled RNA from six plants each. The expression level post infection (2 dpi and 4 dpi) was compared to the expression in uninfected control plants using non-parametric, Mann-Whitney U test. (** = $p \leq 0.01$)

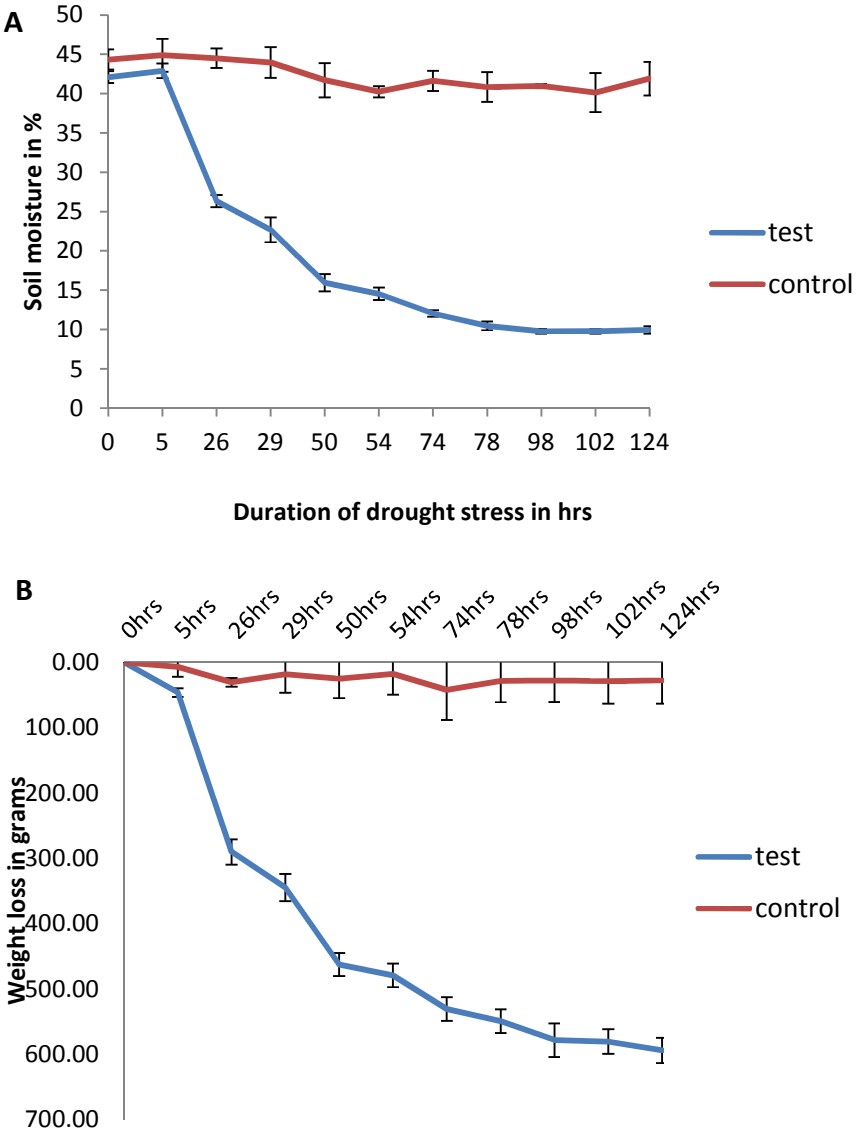


Figure 2-9 Variations in soil moisture and pot weight of stressed and control plants during the course of drought induction.

A. The soil moisture of the stressed plants was measured during the course of drought stress and compared with the control. Error bars represent standard error of the mean. Each data point represents the mean of ten plants.

B. The moisture loss from the stressed plants was determined gravimetrically during the course of drought and compared to control plants. Error bars represent standard error of the mean. Each data point represents the mean of ten plants.

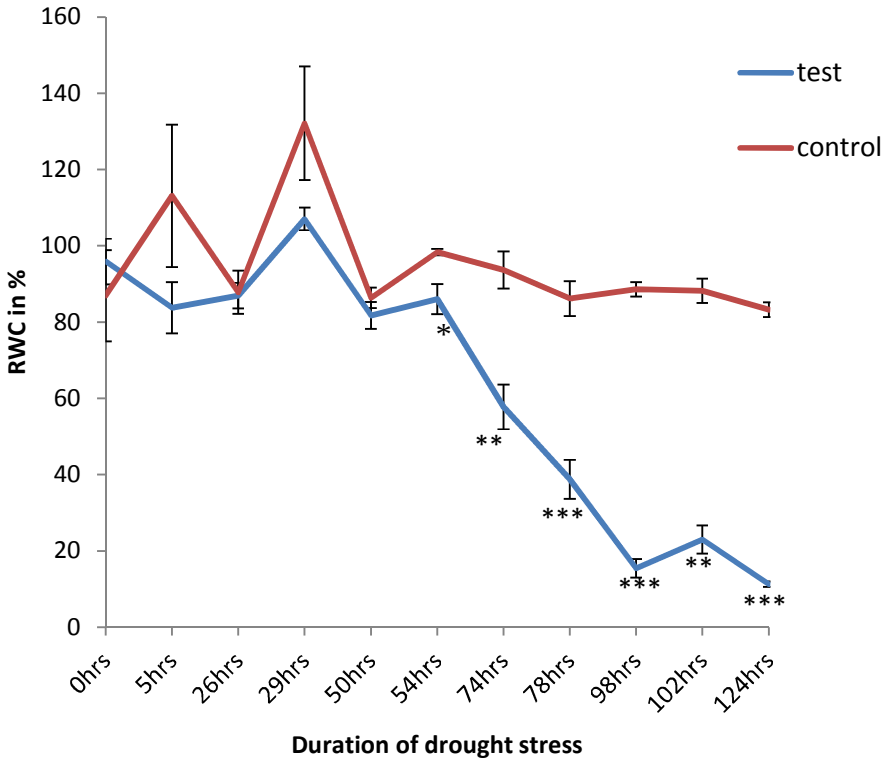


Figure 2-10 The Relative water content of rice leaves during the course of severe drought stress induction.

The relative water content (RWC) of leaves of stressed rice plants and control plants was recorded for the course of drought stress. The RWC of stressed plants was compared with that of the control plants and significance was assigned using the non-parametric, Mann-Whitney U test (* = $p \leq 0.05$; ** = $p \leq 0.01$** = $p \leq 0.005$). Error bars represent standard error of the mean. Each data point represents the mean of 10 plants.

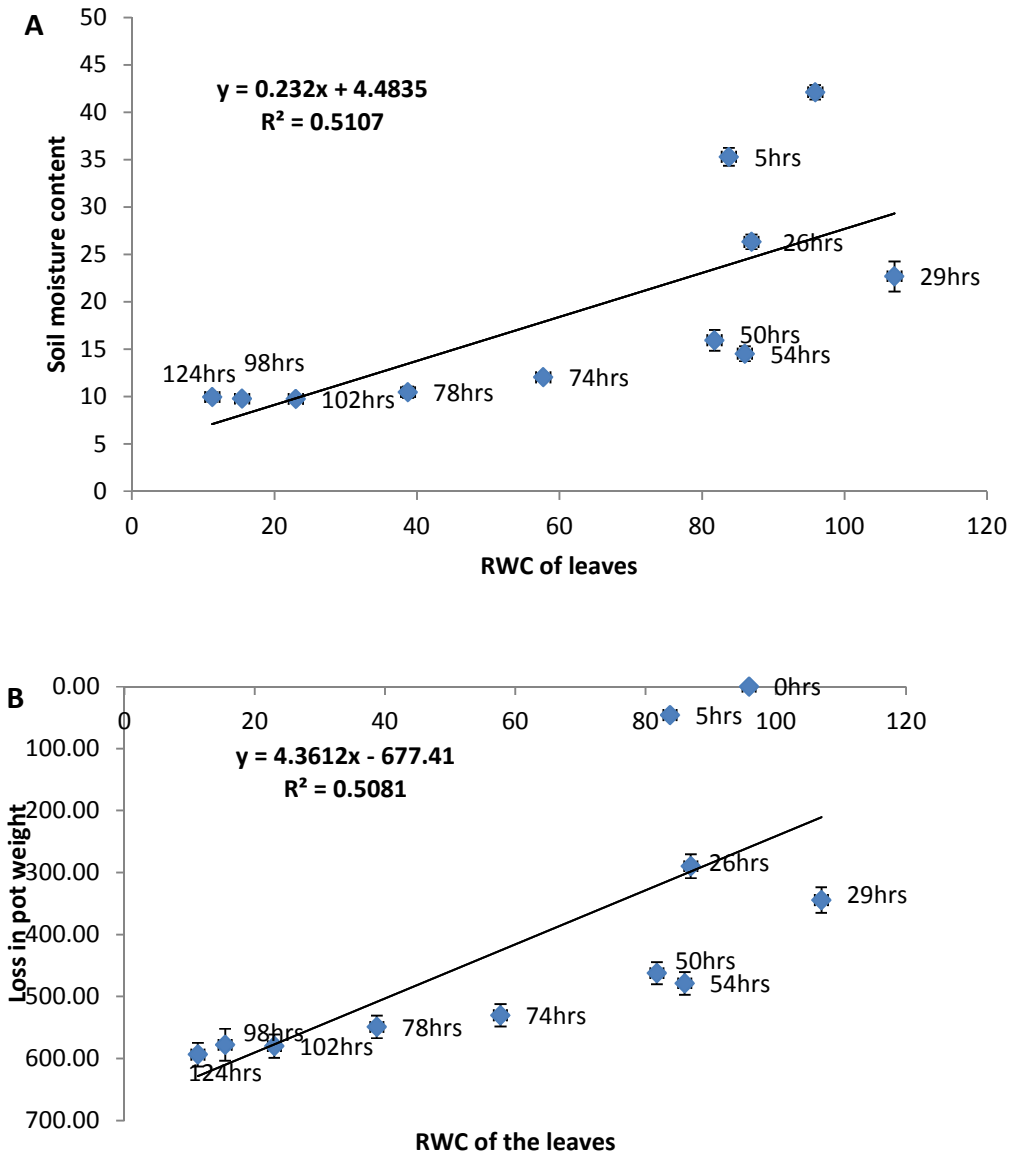


Figure 2-11 Relationship between relative water content and moisture loss during the course of severe drought stress.

A. Soil moisture of the pot was recorded and a leaf was plucked from each plant at the mentioned time points to determine the RWC. Error bars represent standard error of mean for 10 plants.

B. The moisture loss from the pot was calculated by finding the difference in pot weight at these time points, RWC was calculated by plucking a leaf at the same time. Error bars represent the standard error of the mean for 10 plants.

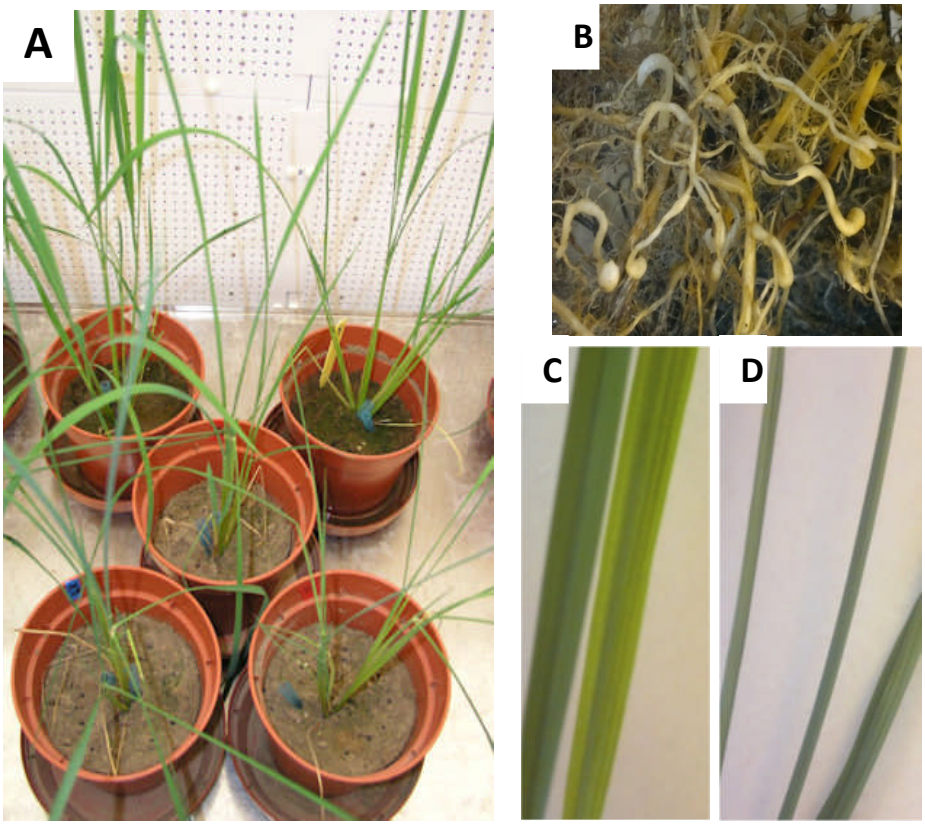


Figure 2-12 The experimental set up and non-destructive stress indicators for drought stress and nematode infection in rice

- **A.** The plants under drought stress, nematode stress and simultaneous multiple stress along with control unstressed plants were grown individually in 6 inch pots standing in saucers. The control and nematode stressed plants were watered from the bottom; water in drought and multiple stressed plants was withheld.
- **B.** The characteristic hooked root galling, produced by *M. graminicola* infection.
- **C.** Leaves of control well watered plants showing no rolling. Leaf rolling score of zero. Leaves of drought stressed plants showing characteristic leaf rolling score of 8 (0-9, 0 - the unrolled leaves and 9- leaves tightly rolled).

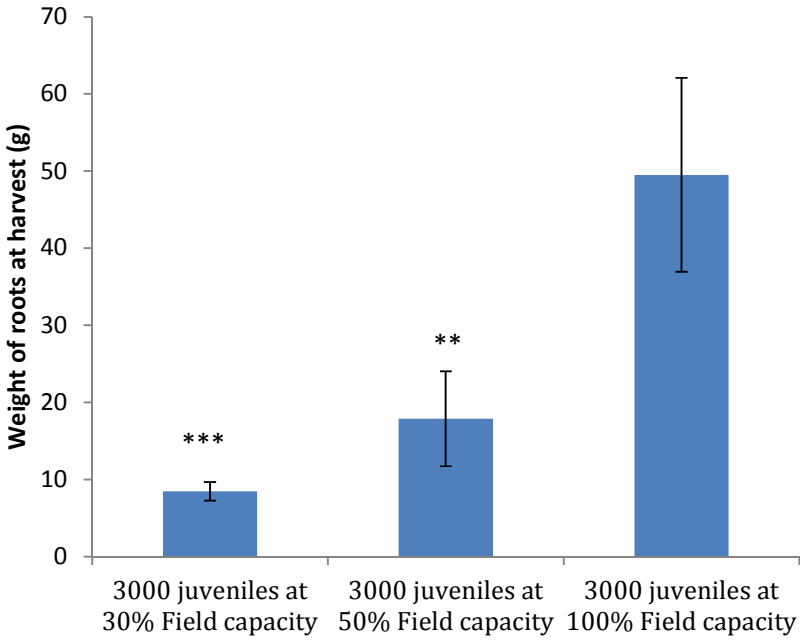


Figure 2-13 Effect of simultaneous root-knot nematode with drought stress under different moisture regimes on root size of the rice plants at tillering stage.

Rice plants maintained at different volumetric soil moisture content (30 %, 50 % and 100 %) were inoculated with 3000 juveniles each and harvested after two generations of RKN infection, the root weight was recorded. The bars represent mean root weight for five plants under the simultaneous stress treatments; error bars represent standard error of mean. The drought stressed plants with 30 % and 50 % of soil moisture content were compared to the well-watered plants (100 %) using the non-parametric, Mann-Whitney U test; * = $p \leq 0.05$.

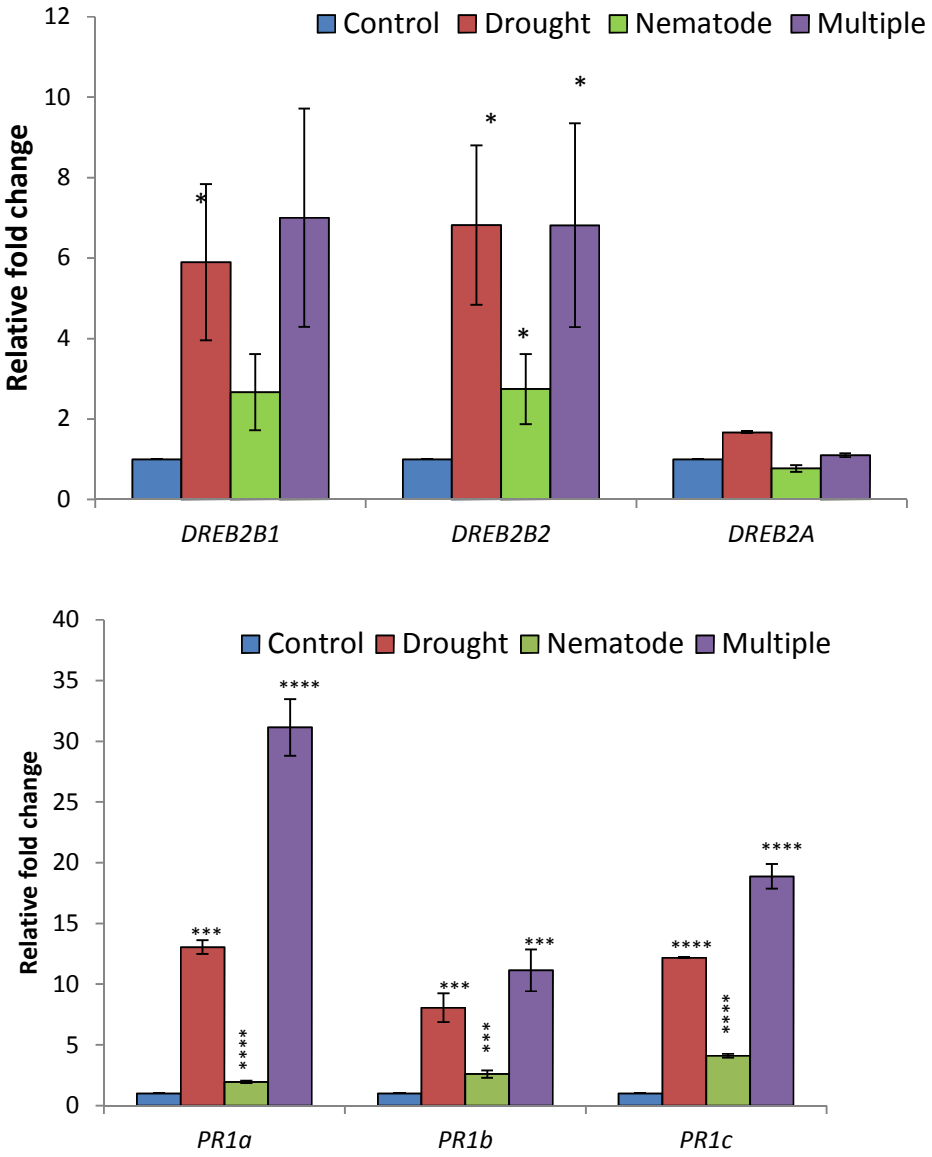


Figure 2-14 Expression analysis of stress responsive genes in roots and leaves of rice plants under drought stress, nematode infection and simultaneous multiple stress

A. Expression levels of drought stress markers, DREB genes under drought, nematode and simultaneous multiple stresses relative to the expression of the corresponding gene in controls. Each bar represents the mean of three technical replicates, with the error bars showing standard error of the mean. The means of stressed plants were compared with the control plants and the significance was determined by Student's t-test. **B.** Expression levels of pathogenesis related protein, *PR* genes under drought, nematode and simultaneous multiple stresses relative to the expression of the corresponding gene in controls. Each bar represents the mean of three technical replicates, with the error bars showing standard error of the mean. The means of stressed plants were compared with the control plants and the significance was determined by Student's t-test. (* = $p \leq 0.05$, *** = $p \leq 0.005$ and **** = $p \leq 0.001$)

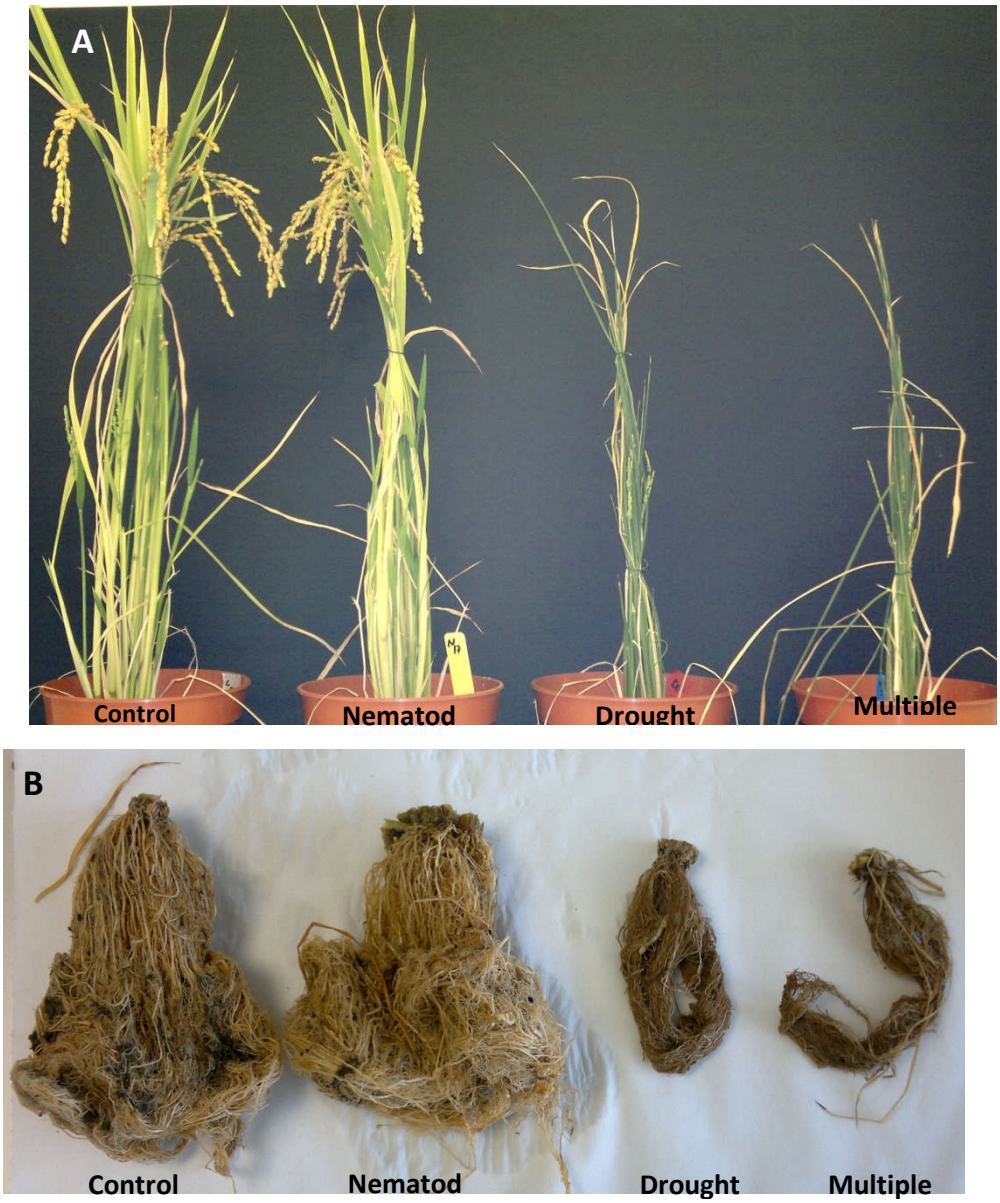


Figure 2-15. Phenotype of rice plants under drought stress, nematode infection and simultaneous drought and nematode stress.

The plants were maintained under the stress regimes for two generations of *M. graminicola* infection and harvested 40 days post inoculation**. A.** Shoots at time of harvest. **B.** Representative root systems post-harvest.

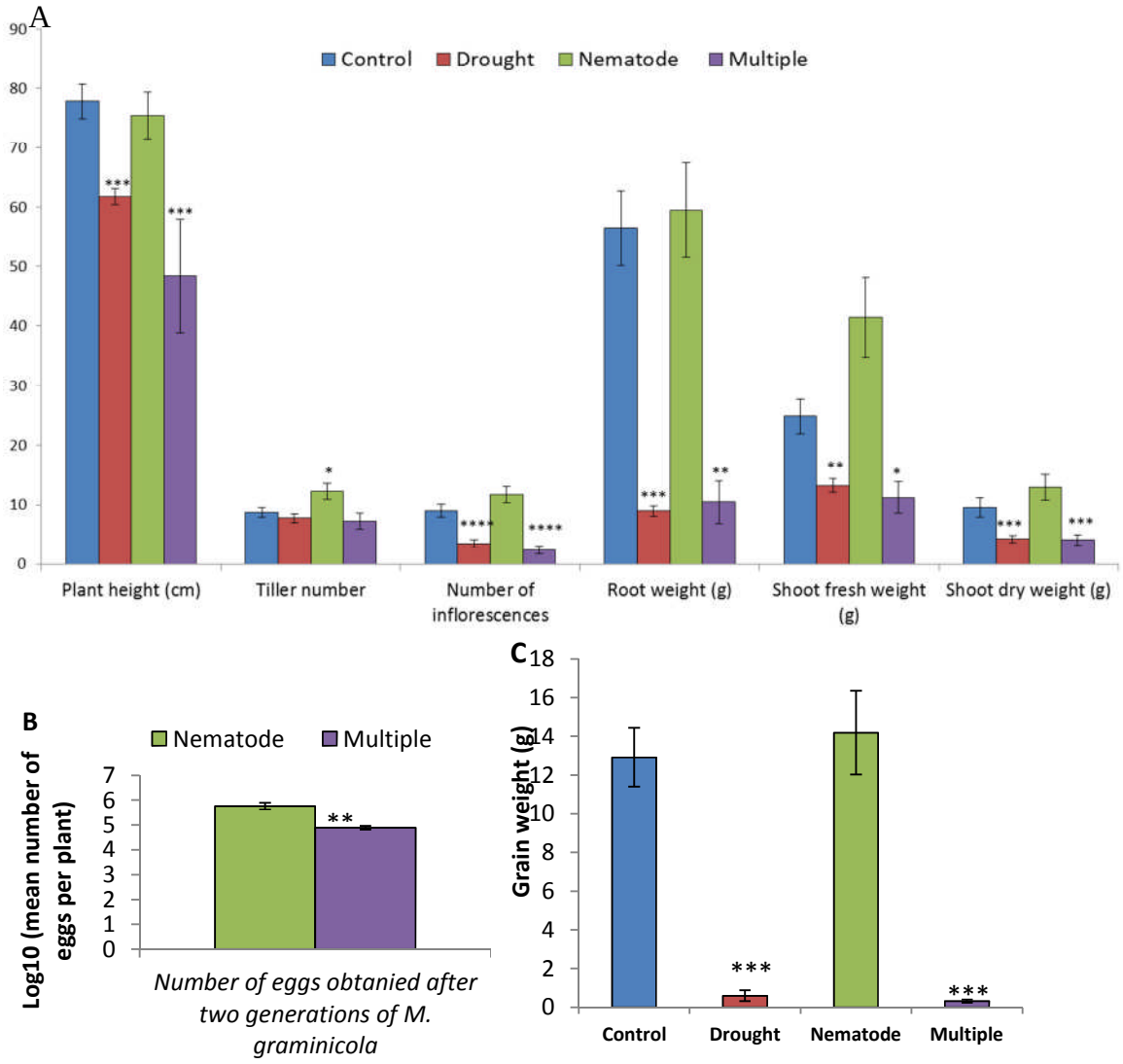


Figure 2-16 . Phenotypic characterisation of rice plants under drought stress, nematode infection and simultaneous drought and nematode stress.

The plants were maintained under the stress regimes for two generations of RKN infection and harvested 40 days post inoculation**. A.** Plant height, tiller number, root and shoot fresh weight, shoot dry weight, number of inflorescences produced were recorded. Each bar denotes the mean of five plants under each infection regime, error bars show standard error of the mean. **B.** Eggs obtained after the two generations of RKN infection on rice plants under nematode stress and simultaneous drought and nematode stress. **C.** Mean weight of grain obtained from plants under different stress regimes. Significance was analysed by Mann-Whitney U test, **** = p≤0.001, *** = p≤0.005, ** = p≤0.01,* = p≤0.05. Means of the stressed plants were compared to the means of control plants for each parameter (n = 5).

2.4. Discussion

2.4.1. Why simultaneous biotic and abiotic stress?

Drought stress accompanied by the RKN *M. graminicola* infection is a devastating combination that limits the productivity of rice plants. Rice, the model, monocot crop plant, experiences drought stresses of different magnitudes in the field at each stage of development. The late vegetative drought stress has a major effect on the gross productivity of the rice crop in all rice ecosystems (Boonjung and Fukai 1996). The dry period in aerobic rice cultivation and in wheat-rice cropping systems especially intensifies *M. graminicola* infestation in fields (MacGowan 1989; Pokharel *et al.* 2007; Jain, Khan and Kumar 2012; Dutta, Ganguly and Gaur 2012). *M. graminicola* presents itself as the most prevalent pest of upland rice in the biggest rice producing belt of South Asia (MacGowan 1989). These two stresses were chosen to study the transcriptomic responses of rice plants to simultaneous biotic and abiotic stresses. A prerequisite for the study was to optimise the two stress treatments, individually as well as in combination, to achieve a successfully replicable stress regime that can mimic field stress. The stress regimes had to be sufficiently intense to produce detectable transcriptomic responses but not be fatal.

2.4.2. Optimisation of RKN infection

A *M. graminicola* colony was obtained from Dr Rosane Curtis, Rothamsted Research, UK and maintained on rice cv Nippponbare. This nematodes were molecularly characterised and confirmed as *M. graminicola* by amplification and sequencing of a diagnostic region of 18S rDNA. The smaller subunit of ribosomal DNA has conserved and variable regions and is a very high copy number gene with a rich database of nematode sequences. The 18S rDNA has been universally used for molecular phylogeny of nematodes (Floyd *et al.* 2002). The characteristic hooked galling further confirmed that the RKN used was *M. graminicola*. *M. graminicola* is known to have a temperature dependent life-cycle; this particular isolate displayed a 20 day life-cycle at 30 ºC on the rice cultivar Nippponbare (MacGowan 1989). The damage caused to productivity and growth of rice plants by *M. graminicola* is proportional to the number of nematodes used as initial inoculum. An initial inoculum density as low as 0.1 J2/g soil is sufficient to generate yield loss of 3.1 % in rice under an upland irrigation

system, whereas a density of 10 J2/g soil can reduce the yield by 97 %. Initial inoculum density of five J2/g soil facilitates the highest J2 and egg density (Poudyal *et al.* 2005). Severity of loss caused by infestation increased with earliness of inoculation time (Jaiswal, Kumar and Singh 2012) . The nematode infection in non-flooded as well as flooded soil also leads to reduction in root mass (Soriano, Prot and Matias 2000). In this study an initial inoculum density of two J2 per gram (3000 J2/ 1500 g soil in each pot) gave replicable induction of pathogenesis-related genes with minimal effect on plant growth parameters.

The *PR* genes are used as markers of systemically acquired resistance (SAR) in plants in response to biotic stress. Twelve members of the PR1 gene family have been characterised in rice, each of these members has a tissue specific, basal expression that changes in response to pathogen attack, wounding and hormone treatment (Mitsuhara *et al.* 2008). The SA-induced *PR1b* gene is generally used as a marker of SAR, induced in rice leaves during fungal, bacterial and pathogen attack (Mitsuhara *et al.* 2008). Our results preliminary experiments show induction of this gene in leaves of plants inoculated with different initial inoculum densities of juveniles (1000-9000 J2s). Thus, we have used the *PR1b* gene as a marker of SAR in shoots to determine the sampling time and tissue to investigate the systemic transcriptomic changes induced by *M. graminicola*. In contrast to the results obtained by us a recent study by Kyndt *et al.* found repression of *PR1b* in shoots of rice plants infected with *M. graminicola* at two early (3 dpi and 7 dpi) infection points.

2.4.3. Optimisation of drought stress regime

Rice production is water dependent and most lowland cultivars of rice require water-logged fields for the majority of their life cycle. The upland rice varieties do not need water-logged fields but their cultivation is still dependent on irrigation. The soil in uplands drains easily and the total available water is less than that available to the lowland rice cultivars with the plants more vulnerable to drought (Boonjung and Fukai 1996). Drought-responsive gene expression changes induced in rice plants have been studied by various groups in tissue culture, by introducing drought using PEG, air drying, withdrawal from hydroponic solutions and application of ABA (Zhao *et al.* 2007; Dubouzet *et al.* 2003; Claes *et al.* 1990). The drought-responsive genes identified by using substrate based artificial drying techniques focus on plant performance under

controlled drought conditions; from an agricultural context, plants may not show similar responses when subjected simultaneously to other biotic and abiotic stresses in the field. Rice crops are prone to vegetative or reproductive drought whereas the laboratory-based stress studies impose drought stress on seedlings. These methods do not replicate the conditions experienced by the plants in soil (Atkinson and Urwin 2012). (Gaudin *et al.* 2013) have discussed the importance of drought screening methodologies for transgenic plants and the need to focus on plant responses in field conditions To mimic the stress experienced in fields it is essential to develop a model that facilitates slow loss of soil moisture. The water status of plants needs to reduce slowly without unnatural acute drying. We have grown rice in 6" polyvinyl pots in loamy sand and placed them in saucers with 1-2 cm of standing water. Severe drought was initiated by withholding water, pots were weighed each day to determine the soil moisture loss and the volumetric soil moisture was also measured. This method of drought stress in soil has been used in proteomic analysis of rice leaves in drought stress and recovery (Salekdeh *et al.* 2002). The stress encountered by the plant needs to be quantified by using various morphological and physiological parameters of plant health to make sure that during the course of the experiment the plants experience the same magnitude of stress each time the samples are taken.

The total amount of water held by soil, as the mass percentage, is termed field capacity (FC). It is an integral property of soil and was traditionally determined as the amount of water held by the soil after the excess gravitational water has drained away (Veihmeyer and Hendrickson 1931). FC can be reliably determined by measuring the moisture equivalent of the soil (Veihmeyer and Hendrickson 1949). Currently the FC can be either directly determined in terms of the volumetric soil moisture content measured using a soil moisture meter or in a traditional way by using gravimetric analysis. It can be used as a direct method of quantifying water deficit in soil. The water potential of soil at any FC value can also be determined using water potentiometers. The soil water potential directly influences the leaf solute potential, water use efficiency, carbon isotope discrimination and RWC of leaves (Impa *et al.* 2005; Govind *et al.* 2009). The drought stress responsive genes in peanut were identified by utilizing the reduced FC as a method of introducing gradual drought stress and by monitoring the stress using leaf water status (Govind *et al.* 2009).

Of the various methods used, RWC gives a direct measure of water content in the plant and was measured using a leaf plucked from drought stressed rice plants. RWC reduced proportionally with the duration of drought experienced by these plants and can be used to quantify the drought stress (Figure 2-11). It is a destructive measurement and cannot be used in a combinatorial biotic and abiotic stress responsive study because of mechanical wounding caused by plucking a leaf. This wounding initiates defence responses similar to those observed in pathogen attack (Zhang and Klessig 2001). In order to use a non-destructive method to quantify the drought stress, correlation between the soil water status and plant water status was established. The soil water status was determined by measuring volumetric soil moisture content and by gravimetric measurements of the pot during the drought stress regime (Jones 2007). The RWC was determined for these plants at each volumetric soil moisture level. The soil moisture content and loss in pot weight decrease proportionally to the RWC of plants during severe as well as gradual water stress experienced by a plant. Thus, the volumetric soil moisture content and gravimetric measurement of pots were used to quantify drought stress experienced by the plants. The drought stressed plants fail to rehydrate and recover if they are stressed beyond their permanent wilting point (Veihmeyer and Hendrickson 1950). The permanent wilting point of cv Nippponbare in the sandy loam topsoil was determined by imposing severe drought. None of the plants that had volumetric soil moisture content less than 20 % recovered after re-watering. Gradual drought was induced by allowing the plants to dry gradually but they were always maintained above 20 % volumetric soil moisture content.

As a preventive physiological response to drought, the rice leaves start rolling to reduce water loss from the leaf surface in the form of transpiration. The extent of leaf-rolling is used as an indirect measure of stomatal resistance and leaf water potential (O'Toole and Cruz 1980). A leaf-rolling score is used to determine the severity of drought stress and to screen drought tolerant varieties of rice (IRRI, Philippines). The unrolled leaves of well-watered plants are given a score of zero and the completely rolled, cylindrical leaves of a severely drought stressed plant are given a score of nine. Together with soil moisture content, the leaf rolling score was used as a visibly indicator of drought stress in rice leaves.

Drought stress influences expression of drought responsive transcription factors that drive the expression of down-stream drought responsive genes. The drought responsive

element binding (DREB) transcription factors interact with the *cis*-acting promoter motifs called drought responsive element (DRE) (Dubouzet *et al.* 2003). The expression of *DREB* genes is induced in response to salinity, cold, heat and drought stress and thus they can be used as the molecular markers of abiotic stress. The *DREB2A* gene and two alternatively spliced transcripts of *DREB2B* show induction under early stages of drought stress in rice leaves (Matsukura *et al.* 2010). The transcripts of *DREB2A* and *DREB2B* in Arabidopsis accumulate specifically in roots of air dried Arabidopsis plants (Nakashima *et al.* 2000). We used induction of these genes in roots of drought stressed rice plants as a marker of drought induction in roots.

2.4.4. Optimisation of simultaneous drought and RKN infection

The transcriptomic response in Arabidopsis induced by a combination of dehydration and infection with the cyst nematode *Heterodera schachtii* has been studied using a laboratory model of plants grown in tissue culture (Atkinson, Lilley and Urwin 2013). But, as established earlier, the plant responses towards a suite of stresses in the field is different from the response of a seedling grown under laboratory conditions. Setting up replicable regimes for induction of these stresses individually and in combination in a soil based experiment was a challenging task. The phytohormones and signalling pathways responsible for stress responses also regulate plant growth and are temporally and spatially expressed in unstressed plants. The tissue to be sampled and time of sampling had to be consistent between all treatments to avoid developmental and tissue-specific change being interpreted as stress responsive changes. Mechanical wounding caused to a plant by plucking a leaf leads to initiation of wounding responses (Schweizer *et al.* 1998). The wounding responses are mediated by the phytohormone jasmonic acid that could interfere with the biotic as well as the abiotic stress responses (Agrawal *et al.* 2003; Halim *et al.* 2006; Niki *et al.* 1998). Thus, only one sample was collected from each plant, to avoid the additive effect of wounding. To explore the transcriptomic changes pertaining to a late vegetative stress drought similar to that experienced in the field, the stress treatments were induced after the plants had reached tillering stage. The volumetric soil moisture content and leaf rolling score were used to induce and monitor drought stress. The systemic changes induced by nematode infection were reproducibly detectible in mature leaves of the plant at an early infection point of 4 dpi. Four days post infection *M. graminicola* juveniles successfully establish

isible feeding site on rice root. The initiation of feeding site is characterised by transcriptomic changes leading to reprogramming and re-differentiation of cells to form giant feeding cell (Roland N. Perry 2009). The 4 dpi is an ideal time point for studying systemic changes in leaves and also to identify local changes in roots. Nematode mobility in soil is affected by the pore size as well as moisture content of the soil (Prot and Van Gundy 1981). The effect of reduced soil moisture content on the rate of nematode infection was determined by reducing soil moisture to 30 % and 50 % and infecting the plants with 3000 juveniles. The plants that were maintained at 50 % of initial volumetric soil moisture content facilitated nematode infection without compromising the root size. Thus, it was decided to maintain the plants at 50 % reduced soil moisture for successful induction of simultaneous drought and nematode stress. To mimic the progressive prolonged drought stress in late vegetative phase, the plants were maintained at 50 % soil moisture for ten days before being infected by the RKN. This overlaps with the drought and infestation pattern observed under aerobic rice cultivation in soil where the seedlings are transplanted in fields under puddle conditions and irrigated thereafter. The following dry period intensifies nematode infection. The root and shoot sampling was done 4 dpi for all treatments. Figure 2-17 shows a schematic representation of the devised model system to be used for transcriptomic analysis for inducing drought and RKN stresses individually and in combination. The drought and nematode stress regimes showed phenotypic changes similar to those observed in plants under similar stress in fields. We have successfully established a model of inducing dual (drought and nematode) stress on rice cv Nippponbare in soil.

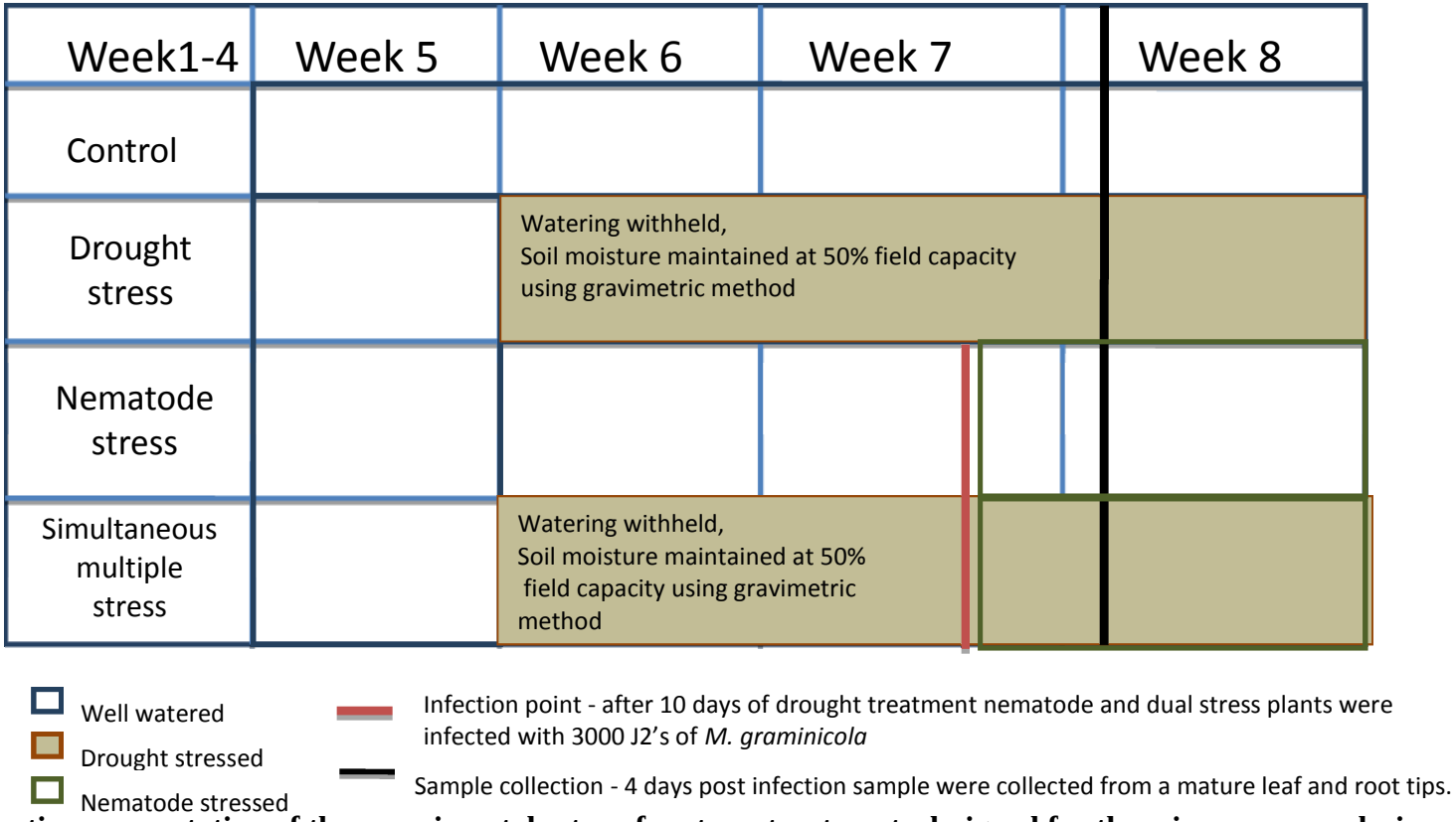


Figure 2.17 Schematic representation of the experimental set-up for stress treatments designed for the microarray analysis.

The plants were divided into four treatment groups. The three week old seedlings were transplanted into 6 inch pots and all four sets were well watered and grown in similar conditions without infection. At the beginning of the fifth week water was withheld in the drought and dual stresses and maintained at 50 % of field capacity thereafter. After ten days the plants in the nematode and dual stress groups were infected with 3000 J2s each. The root and mature leaf samples were collected and snap frozen for all four treatments four days post nematode infection.

3 Chapter

Transcriptomic response of rice to simultaneous biotic and abiotic stress

3.1. Introduction

Stress responses in plants are the result of complex interplay within vast arrays of genes directly and indirectly responsive to the stress experienced. The plant responses to a single biotic or abiotic stress have been well studied and understood. Responses of rice to a range of environmental stresses in various ecosystems have been explored and exploited to develop better performing cultivars. However, the crops in the field are more likely to experience simultaneous biotic and abiotic stresses at any given point in time. Plant responses under such situations are hard to speculate as the pathways involved in singular stresses interact in an unknown fashion to formulate the resultant response towards simultaneous stresses (Atkinson and Urwin 2012). For example, the abscisic acid-mediated abiotic stress-specific response works antagonistically to the salicylic acid signalling which is specific to biotic stress in plants. The response to simultaneous biotic and abiotic stress in a plant is a result of the fine-tuning between various hormonal and signalling pathways.

3.1.1. Microarray technology as a tool of transcriptomic analysis

The post-genomic era poses the challenge of functional characterisation of structurally analysed genomes. After the rice genome had been sequenced in 1998 the ultimate goal was to assign function to the sequenced genes. Along with traditional reverse genetic techniques, microarray technology was used as the robust tool for understanding comprehensive gene expression at a genome wide level (NIAS 2001). Since its first mention in 1995, for nearly two decades microarray has undoubtedly been one of the most efficient and widely used techniques in the field of biology and medicine. Beginning from the DNA arrays it has grown into lipid, carbohydrate and protein arrays based on similar technologies (Nuber 2005). A typical DNA microarray refers to a collection of various ssDNA sequences fabricated on a surface in a grid like pattern. The ssDNA fragments fabricated on the array are termed probes or sequence tags depending on their length and the chemical nature of these probes.

3.1.1.1. Milestones in development of microarrays

Initial high-density arrays comprised DNA sequences 200 bp in length, spotted by a robotic spotter on a poly-lysine coated microscope slide. All cDNAs to be spotted on to the array were synthesised, prior to spotting, and this often involved cloning and PCR amplification (DeRisi *et al.* 1996). The biggest drawback of these spotted arrays was the non-reproducibility of similar arrays that made inter-array comparison difficult and would lead to false results due to variations from array to array (Draghici 2010). Spotted arrays were replaced by more complex and sophisticated higher density arrays called *In-situ* synthesised arrays that have sequence probes synthesised directly on to the arrays. Leading companies have developed and used different technologies to commercially produce these arrays for research as well as biomedical purposes. Affymetrix (Santa Clara, USA) generated a large catalogue of expression DNA arrays by light-directed spatially addressed chemical synthesis of oligonucleotide probes on to the array with photolithography (Wodicka *et al.* 1997; Lockhart *et al.* 1996). Affymetrix rival Agilent (Santa Clara, USA) uses inkjet technology where a printer head deposits specific nucleotides where required (Blanchard, Kaiser and Hood 1996). The inkjet printers had four cartridges one for each phosphoramidite nucleotide which was delivered onto a hydrophilic region of a glass slide surrounded by a hydrophobic area. This was patented and is used for production of low volume custom arrays (Hughes *et al.* 2001). Another method involving electrochemical synthesis of the sequences, base by base, by activating an electrode embedded in the surface according to predetermined sequence, has been used by Combimatrix (Mukilteo, USA) for preparing custom arrays for biomedical purposes (Draghici 2010). Illumina (San Diego, USA) developed high density optical sensor arrays where the DNA was synthesised on a microsphere that is placed on a fibre optic array in micro-wells (Michael *et al.* 1998).

3.1.1.2. Data analysis and identification of differentially expressed genes

Analysing the vast amount of data generated by a whole genome microarray experiment presents a daunting challenge to researchers. A successful microarray experiment is a well-designed systematic study with a defined aim, adequate biological replicates of considered treatments, use of a standardised microarray protocol, robust yet simple statistical analysis and representation of data in a universally understandable and acknowledgeable format. The main parts of a microarray study are experimental design,

image acquisition, data pre-processing and normalisation, identification of differentially expressed genes (DEGs) and exploratory data analysis (Leung and Cavalieri 2003).

Pre-processing for any array involves background removal, normalisation of probe intensities; in Affymetrix arrays it also includes probe intensity summarisation (Mohapatra and Krishnan 2011). Majorly used algorithms are Robust Multichip Averaging algorithm (RMA), GCRMA, Li-Wong method and the probe logarithmic intensity error (PLIER) and modified versions of these.

Due to various systematic and biological variations that can occur during a microarray experiment, identification of DEGs based on an expression cut-off can recognise many false positives and is not efficient. Thus assigning statistical significance to the expression value for any gene and then using a probability cut-off to reject the null hypothesis that the gene is not differentially expressed is a more acceptable method (Leung and Cavalieri 2003).

The DEGs have to be annotated and functionally characterised. This opens a vast field of array informatics that deals with 'the post gene list' challenges and helps in systematic use of the published literature and online resources in assigning biological importance to the identified genes and accessing data obtained from microarray studies (Geschwind *et al.* 2002). A large number of bioinformatics tools are available for analysis and comprehension of microarray data (Koschmieder *et al.* 2012). Due to the vast range of available platforms and variability in the data analysis, it has been hard to present the microarray data from any study in a manner useful to a broader scientific community. Thus a guideline of Minimum Information About a Microarray Experiment (MIAME) has been developed to standardise the process (Brazma *et al.* 2001).
From array design to the functional characterisation of the identified genes, microarray provides flexibility and variability because of which it can be used for accomplishment of a wide range of biological aims, and hence the complexity in planning and presentation of the data.

3.1.2. Rice arrays

Several different rice arrays have been developed so far, each of which has a different design and variable coverage of the rice genome. The 20K and 45K coverage of rice genome long oligonucleotide arrays were developed by the University of California at

Davies (UCD) The Institute for Genomic Research (TIGR) and Iowa State University (ISU), and are funded by the National Science Foundation, Plant Genome Programme. These arrays are no longer publically available, but have been widely used in the past for rice transcriptomic studies. Agilent also developed the 22K and 44K coverage Rice gene chips. The 44K chip has 60-mer oligonucleotide probes corresponding to Rice Annotation Project (RAP) loci with transcripts based on rice full-length cDNA (representative cDNA), transcripts with expressed sequence tag (EST) support, as well as transcripts based on *ab-initio* gene prediction from the japonica cultivar group. The 57K rice genome array developed by Affymetrix through the GeneChip® consortia programme provides a comprehensive coverage of the rice genome with 51,279 transcripts; 48,564 from the japonica cultivar group and 1.260 from the indica cultivar group. The array has been constructed to the standard Affymetrix protocol with 11 probe sets per transcript each of which is a 25-mer oligonucleotide probe. The sequences are obtained from UniGene build 52 (7th May 2004), GeneBank® mRNAs (13th July 2004), TIGR osa1 version 2.0 release gene predictions, and the International Rice Sequencing Project.

3.1.3. Transcriptomic study of drought and dehydration stress in rice

The first large-scale expression analysis of abiotic stresses in rice was performed using two-colour spotted arrays prepared by spotting 1,700 ESTs from cDNA libraries generated from stressed tissues to study drought, cold, high salinity and ABA response (Rabbani *et al.* 2003). Other drought responsive genes have been identified by studies involving hormone application, air-drying or polyethylene glycol induced stress. However, these methods are not actually representative of drought stress experienced in the field (Yazaki *et al.* 2003; Yazaki *et al.* 2004). Studies with a more realistic approach have been performed by generating ESTs from drought stressed rice grown in pots under upland growth conditions (Reddy *et al.* 2002). A global expression analysis of spatial responses under drought and high salinity was conducted using a genome wide 70-mer array focusing on shoot, panicle and flag leaf of the plant. The organ specific drought responsive transcriptomic changes were observed with little overlap between the expression in different tissues (Zhou *et al.* 2007). Comparison of drought responses in tolerant and susceptible cultivars has been important in revealing novel response elements. A transcriptomic analysis was performed using the 20k NSF oligonucleotides

array to investigate effect of long-term drought on tolerant and susceptible rice cultivars. Most of the genes this study characterised are involved in senescence and degradation of the plant (Degenkolbe *et al.* 2009).

Comprehensive temporal and spatial expression profiling of drought responsiveness of a drought tolerant line, obtained from a cross between two drought tolerant IR64 introgression lines, was performed using the Affymetrix rice GeneChip array (Fujita *et al.* 2010). This was the first study to look at the root-specific drought responses along with the leaf and panicle at the major stages of tillering, panicle initiation and booting. Fujita *et al* reported a tissue specific transcriptome response to drought. Another genome wide study used the same Affymetrix rice GeneChips, to investigate the differences in drought responsiveness of a drought-tolerant indica cultivar Nagina N22 and the high-yielding drought-susceptible cultivar IR64. A genotype-specific drought response was identified in whole seedlings of these two cultivars (Lenka *et al.* 2011). Another similar study identified root specific responses in two near isogenic lines of IR64 with ~97 % similar background but contrasting drought tolerance properties. This study used soil-grown plants, mimicking field conditions and the 44K Agilent genome array to explore drought responsive transcriptomic changes. The study showed that there can be different drought tolerance mechanisms in near isogenic lines (Moumeni *et al.* 2011).

3.1.4. Transcriptomic study of nematode stress in rice

Sedentary plant parasitic nematodes dramatically re-programme host cells to convert them into elaborate feeding sites that act as the nutrient sinks required for successful and sustainable parasitism. Plants respond to nematode invasion locally as well as systemically (Sijmons, Atkinson and Wyss 1994). The transcriptomic responses of rice and other monocotyledon plants towards nematode infection have not been studied in detail but the responses of various dicotyledonous plants towards nematode infection have been well studied and documented (Kyndt *et al.* 2012a). The localized plant response towards RKN has been explored by dissecting galls or micro-aspirating contents of giant cells of infected plants (Fuller *et al.* 2007; Szakasits *et al.* 2009; Bar-Or, Kapulnik and Koltai 2005; Wang, Potter and Jones 2003) in order to carry out transcriptomic analysis. The nematode induced systemic responses in plants have been studied by transcriptomic analysis of whole root systems (Puthoff *et al.* 2003; Hammes

et al. 2005; Barcala *et al.* 2010; Das *et al.* 2010; de Sá *et al.* 2012). Transcriptomic reprogramming in the feeding sites of *Meloidogyne graminicola* infecting rice roots was recently investigated by mRNA sequencing of gall tissue at 3 dpi and 7 dpi. The same group also studied differences in the systemic changes induced during infection by either *M. graminicola* or the root-rot nematode *Hirschmaniella oryzae* using quantitative PCR. The systemic defence responses initiated by the migratory nematode and the RKN were similar in nature. This study provides a valuable insight into the systemic defence responses during *M. graminicola* infection but a genome wide transcriptomic analysis of the systemic responses is required to explore the missing links in the story (Kyndt *et al.* 2012b).

3.1.5. Transcriptomic study of simultaneous biotic and abiotic stresses in rice

Being sessile, plants are subject to a combination of simultaneous environmental stresses in the field. Evidence suggests that the response towards a pair of simultaneous biotic and abiotic stress is not always additive of the responses seen towards these stresses individually. Plants treat each set of simultaneous stress as a different environmental condition and tailor their response specifically to it. This may involve differential regulation of a new set of genes that were not being induced or repressed by any of the stresses individually and *vice versa* (Mittler 2006). The response can be non-additive, additive or interactive depending upon the nature and extent of damage caused by either of the two stresses (Atkinson and Urwin 2012). There is very limited knowledge available to address the effect of simultaneous biotic and abiotic stress in plants; there is no systematic whole genome transcriptomic study conducted on rice to explore how the plant responds to simultaneous stress (Hewezi, Léger and Gentzbittel 2008; Rizhsky, Liang and Mittler 2002; Luo *et al.* 2005). The only study involving both nematodes and water stress was conducted to explore water status and growth in upland rice suffering the combined effect of cyst nematodes and drought, but it did not investigate the molecular aspects of the combined response (Audebert *et al.* 2000). Most of the genes recognised as ‘key players’ in biotic as well as abiotic stress responses in rice were identified by comparing the DEGs obtained by two independent studies focusing on singule stresses individually (Jain, Ghanashyam and Bhattacharjee 2010; Li *et al.* 2006a; Narsai *et al.* 2013). Transcriptomic studies in other crop plants have shown

that simultaneous stresses regulate a set of genes that are not involved in single stress, the effect is non-additive and in the case of herbivore damage, can lead to long term changes in the transcriptome that can act as immunological memory (Atkinson and Urwin 2012; Luo *et al.* 2005). Thus, it is hard to predict the response towards simultaneous stresses by comparing the transcriptomic studies looking at these stresses individually. There is a crucial need for a systematic study to investigate the effect of simultaneous multiple stresses on the same plant.

To understand these responses it is essential to explore genome wide expression changes rather than focusing on any individual signalling pathway or gene. A microarray experiment was performed to investigate genome wide transcriptomic changes in rice towards simultaneous vegetative drought and RKN infection. Figure 3-1 provides a schematic representation of the microarray experimental design.

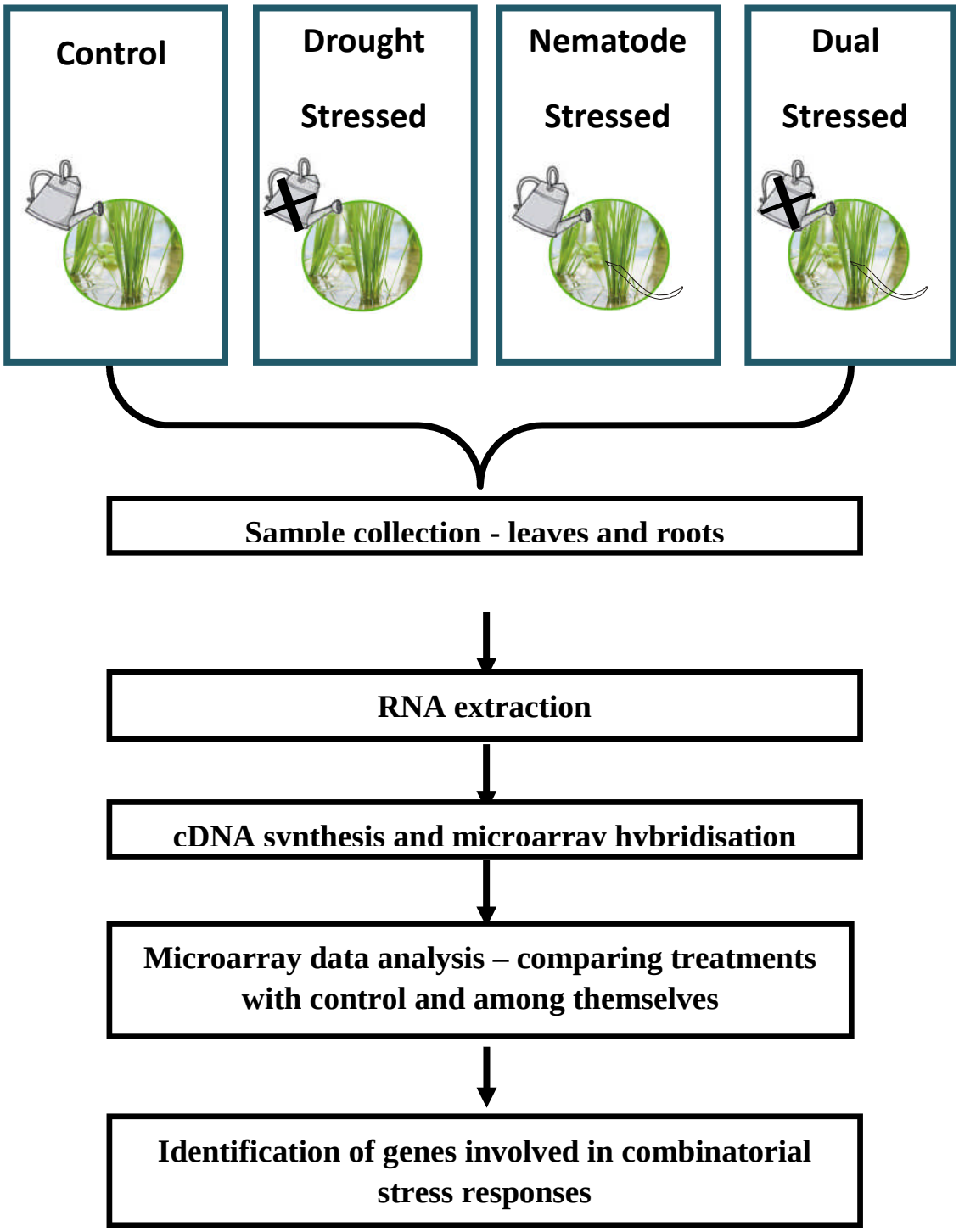


Figure 3-1 The experimental plan for determining transcriptomic responses in rice under simultaneous biotic and abiotic stress.

The experiment comprised of four treatments A- well watered and uninfected control plants, B-drought stressed plants, C- nematode infected plants and D-simultaneous drought and nematode stressed plants. RNA obtained from leaves and roots of each treatment type was used to synthesis cDNA that was hybridised on the GeneChip® Rice Genome Array.

3.2. Materials and methods

3.2.1. Stress treatments

The wild type rice (*Oryza sativa, cv* nipponbare) seeds were sterilised and germinated in Petri dishes as described in Section 2.2.1 One week old germinated seeds were transplanted into 24 cell trays and were left standing in 2-3 cm of water in gravel trays (Section 2.2.2.). Four weeks later seedlings were transplanted in six inch pots with pre-positioned pipette tips and GF/A filter paper as described in chapter 2.2.5. The plants were maintained at a temperature of 28 °C with 80 % humidity for 11 hours during the daytime and at 22 °C with 80 % humidity for 13 hours during the night in the Fitotron (Leicestershire, UK) standard growth chamber at light intensity of 350 µmol m^{-2} s^{-2}. The soil moisture was monitored each day using a soil moisture meter and evapotranspiration was monitored using the gravimetric measurements. The volumetric soil moisture content of each pot at saturation was measured and is referred to as initial volumetric soil moisture content. The plants were allowed to establish in the bigger pots for one week, well watered and unstressed. The plants were divided in four treatment sets; **A** - control (well watered and uninfected), **B** - drought stressed (withheld watering and uninfected), **C** - nematode stressed (well watered and infected with *M. graminicola*) and **D** - simultaneous multiple stressed (simultaneously drought and nematode stressed). Each treatment group comprised of ten plants. The pots were arranged randomly in controlled temperature chambers (Figure 2-1B). After the one week establishment period watering was withdrawn for plants in treatment sets B and D. Once the soil moisture reached 50 % of the initial volumetric soil moisture content and the plants started to exhibit visible symptoms of drought stress, i.e. leaf rolling (score $\geq$ 6), the plants were watered each day with the equivalent amount of water that the plant transpired in the previous 24 hours. These plants were maintained at 50 % of initial volumetric soil water content for a week. After that time, the plants in treatment sets C and D were infected with 3000 second stage juveniles of *M. graminicola.* The juveniles were extracted as described in Section 2.2.4. Approximately 1000 juveniles were introduced into each of the three pipette tips inserted into the pot. The nematode infection was performed immediately after daily watering to facilitate movement of the nematodes in the drought stressed soil.

3.2.2. Tissue sampling

Leaf and root tissue sampling of the experimental plants was carried out four days post nematode infection (dpi). Samples were collected from ten plants per treatment. A mature leaf, the tallest leaf of a tiller other than the main tiller, was picked and frozen in liquid nitrogen. The roots were washed in water to remove soil and then 1 cm of the root tip was collected per plant and snap frozen. Following sample collection, plant height, fresh weight and dry weight of the roots and the shoots plus the tiller number was recorded for each plant. Three biological replicates of the complete experimental set-up were performed in the controlled temperature room over a period of six months in 2011.

3.2.3. RNA extraction

A pooled total RNA extraction was performed using the RNeasy plant mini kit (Qiagen, place). Tissue from the ten plants of each treatment in a biological replicate was pooled and ground in the frozen state using liquid nitrogen. 100 mg of this ground tissue was used for each RNA extraction. In total 24 RNA samples were prepared (4 treatments x 3 replicates x 2 sample types - roots and leaves). RNA was quantified and its purity assessed using a Nanodrop1000 spectrophotometer (Thermo Scientific, Wilmington, USA) according to the manufacturer's instructions. The integrity of the RNA extracted was analysed using a 2100 Expert Bioanalyzer (Agilent Technologies, Santa Clara, USA), according to the manufacturer's instructions.

3.2.4. Sample preparation for microarray hybridisation

10 µl of RNA from each of the above 24 samples was sent to Nottingham Arabidopsis Stock Centre (NASC, Nottingham, UK) for microarray analysis. The samples were re-analysed on a Bioanalyzer to check the RNA integrity after transportation. Anti-sense RNA preparation was carried out using the GeneChip® 3' IVT Express Kit (Affymetrix, Santa Clara, USA), as per the manufacturer's instructions. The process has been summarised in a flow chart (Figure 3-2). For monitoring the hybridisation efficiency, RNA samples were spiked with *in vitro* synthesised poly-adenylated transcripts for four *B. subtilis* genes not found in eukaryotes. The anti-sense RNA was hybridised on the GeneChip® Rice Genome Array (Affymetrix, Santa Clara, USA). The

prokaryotic genes bioB, bioC, bioD involved in biotin synthesis from *E. coli* and Cre from bacteriophage P1 are spiked in staggered concentration directly in the hybridisation control to assess hybridisation efficiency. A cocktail of poly-A tailed *Bacillus subtilis* transcripts *lys*, *dap*, *thr*, *trp* and *phe* is also spiked into the total RNA. The final concentrations of the controls, relative to the total RNA population, are: 1:100,000; 1:50:000; 1:25,000; 1:7,500, respectively. The hybridisation intensities of these controls on the gene chip array monitor the labelling efficiency and the data quality. The internal endogenous *Oryza* genes, GAPDH, 18S rRNA, 25S rRNA and 5.8S rRNA are used to assess the quality of RNA.

3.2.5. Microarray data analysis

The .CEL files containing raw probe intensity values were obtained from NASC and analysed using GeneSpring GX 12.1 software (Agilent Technologies, Santa Clara, USA). The data were normalised using a base transformation to median of all files, for each probe the median of the log summarised value from the sample was calculated and subtracted from each sample. The data was subjected to the PLIER16 probe summarisation algorithm and quantile normalisation. It runs an optimisation procedure that ensures the difference between PM (perfect match) and MM (mis-match) probes is non-negative; this also leads to near zero values for absent probes (Therneau and Ballman 2008). To generate a probe summarisation these values are spiked with a constant (16) followed by logarithmic transformation (Affymetrix 2005). This maximises the sensitivity for low expression. The normalised probe sets were filtered on their signal intensity. Probe sets with a signal intensity value of less than 20 % of the maximum signal intensity in any one of the twelve samples were removed from the analysis. A one-way ANOVA was performed on the remaining probes; a Student-Newman-Keuls post hoc test was performed along with Benjamini-Hochberg simultaneous multiple test correction. Genes showing a positive or negative two-fold expression change and a corrected *p*-value of ≤ 0.05 were designated as DEGs. The lists of genes that were differentially expressed in each treatment relative to the control were compared to identify the unique responses and the overlaps between the various stress treatments.

3.2.6. Validation of microarray results by quantitative RT-PCR

A selection of DEGs with a range of positive and negative expression values across the three treatments was made separately for roots and leaves. The mRNA sequences for these genes were obtained from the Rice Genome Annotation Project (RGAP) (http://rice.plantbiology.msu.edu/index.shtml). Primers for qPCR were designed using the latest version of Primer3plus web interface (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) with standard qPCR settings; Tm = 60 °C ± 1 °C, GC content 50 – 60 %, primer length ~20 nucleotide, and product length of 100-150 bp, with primers spanning an intron. The primers were synthesised and obtained from Sigma Aldrich (Table 7 and Table 8).

Total RNA was extracted from the pooled tissue samples of each treatment for all three biological replicates. An on-column DNase treatment was performed during each RNA extraction using RNase-free DNase kit (Qiagen) using manufacturers instructions. Equal quantities of RNA from each replicate were pooled together to provide 1µg of total RNA in a first strand cDNA reaction performed using Superscript II reverse transcriptase (Invitrogen) with an anchored oligo-dT primer according to the manufacturer's instructions. The quantitative RT-PCR (qRT-PCR) was performed using the reagents and the cycling programme detailed in Table 4 and Table 5, respectively. Reactions were performed and analysed using an Mx3005P instrument (Stratagene). A 5-fold or a 10-fold dilution series of cDNA were used to determine the efficiency and specificity of each primer pair. Specificity was analysed by performing a dissociation curve at the end of each cycling programme. Primers with an efficiency of 100 ± 10 % and an R^2 value ≥ 0.98, amplifying a single product were chosen and used for each gene. Figure 2-2 and Figure 2-3 show representative standard curve, amplification plot, dissociation curve and amplification plot in control and all three treatment samples for the Tumor protein homolog (*TPH*), LOC_Os11g43900.1, which was used to normalise the expression of the target genes across the treatments. The relative starting quantity of cDNA was determined by recording the number of cycles needed for the sample to reach the threshold fluorescence compared to the normalising gene. The relative quantity of a transcript in samples from different stress treatments was determined by comparing to the expression in the un-stressed plants (control) relative to the expression of normalising gene in corresponding stress treatment.

The expression of target genes was analysed using 5 µl of 5-fold diluted cDNA synthesised in a 20 µl reaction using 1 µg of RNA. The qRT-PCR was carried out using the reagents and cycling programme detailed in Table-3. Three technical replicates were performed for each reaction. The transcript expression in each treatment was compared to the control plants to determine the log2 fold change in expression due to the stress treatments. The log2 fold change in expression of transcript determined by qRT-PCR was plotted against the log2 fold change values obtained from microarray analysis and the correlation coefficient was calculated for each treatment.

3.2.7. Functional analysis of genes differentially regulated

Gene ontology enrichment analysis was performed on the significantly differentially regulated genes using singular enrichment analysis (SEA) by agriGO. The genes were grouped according to the biological processes they belong to. The biological processes significantly over-represented in the list of DEGs for each treatment, as compared to the whole chip, were represented as a flow chart, colour coded with the level of significance.

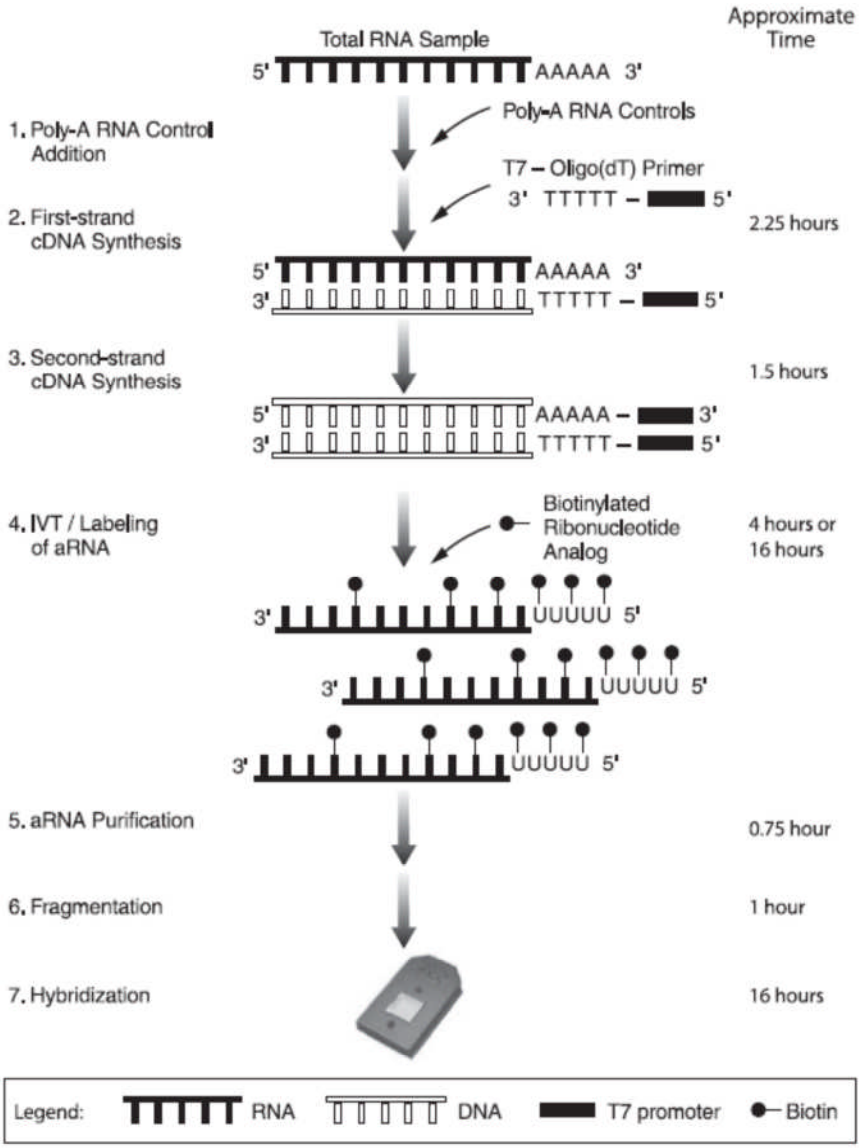


Figure 3-2 Overview of the GeneChip® 3' IVT Express Kit Labelling Assay

1. Poly-A RNA controls were added to the total RNA sample for quality control. 2. Reverse transcription to synthesise First-Strand cDNA is primed with T7 oligo (dT) primer to synthesise cDNA containing a T7 promoter sequence. 3. Second-Strand cDNA synthesis converts the single-stranded cDNA into a double stranded DNA (dsDNA) template for transcription. The reaction employs DNA polymerase and RNase H to simultaneously degrade the RNA and synthesize second strand cDNA. 4. *In vitro* transcription to synthesise biotin-modified aRNA with IVT labelling master mix generates multiple copies of biotin-modified aRNA from the double stranded cDNA templates; this is the amplification step. 5. aRNA purification removes unincorporated NTPs, salts, enzymes and inorganic phosphate to improve the stability of the biotin-modified aRNA. 6. Fragmentation of the labelled aRNA prepares the target for hybridization to GeneChip® 3' expression arrays (adopted from the GeneChip® 3' IVT Express Kit user manual from Affymetrix).

Gene Locus	RGAP annotation	Forward primer	Reverse primer	Product length (bp)	Efficiency (%)
LOC_Os03g62670	S-adenosylmethionine-dependent methyltransferase/ methyltransferase/ thiopurine S-methyltransferase	AGTGCTCGATTACAAGGAGGTG	TCCACCTTGCGATTTTCTCC	113	105.7
LOC_Os01g72330	OsRR4-Rice type-A response regulator	AACAGGTGCTTGGAAGATGG	TTTGTGTGGCGGCTTATCTG	144	96.2
LOC_Os03g59360	Remorin, putative	TCAACAACCGGTTCAAGAGG	TCGTCCAGCTTTCTCTCGATC	106	99.2
LOC_Os06g28630	Expressed protein	ATCGGCCGCATTGATTCAAC	TGAAGTTGGTGGAGCTAGCTG	145	98.2
LOC_Os11g29720	Cytochrome P450 78A4	TATGGTTTGGCCGGATGTTG	TTGCTACGAGGAACTGCATG	127	103.5
LOC_Os02g02830	Ubiquitin-conjugating enzyme E2	TGGCATTCAAGACCAAGGTG	AACCTTGGAGATGGTGAGAGC	107	112.6
LOC_Os01g40094	Protein phosphatase 2C	TTGGTGTTCTTGCCATGTCG	TTCGTCATCCTTTGCTCGAG	104	105.5
LOC_Os04g51610	Calcium-transporting ATPase, plasma membrane-type	ACTGCAGGCGCTTATTGTTG	TTTCCCACAAATGCCAACGG	123	102.1
LOC_Os11g43900	*TPH* (normaliser)	CATTGGTGCCAACCCATC	AAGGAGGTTGCTCCTGAAGA	113	100.3

Table 7 List of genes and gene specific primers used for qPCR-based validation of leaf microarray results.

The gene loci correspond to Osa1 (MSU v7) loci obtained from ricechip.org. The primers were designed using Primer 3 web interface with qPCR suite and checked using BLASTn for homology to other known rice ESTs. Primer efficiencies were determined by producing standard curves for each primer pair at varying cDNA concentrations using MxPro software (Stratagene).

Gene locus	RGAP annotation	Forward primer	Reverse primer	Product size	efficiency
LOC_Os02g04780	Expressed protein	ATCGTGATGGGGAAGAAGAGC	TTGAGCGCGTCTTTGATGTG	113	116
LOC_Os03g12510	Non-symbiotic hemoglobin 2	TGAAGAAGGATTCCGCCAAC	TCTTGAGCTTGGGGTTCTTCTC	132	92.4
LOC_Os01g52110	RING finger and CHY zinc finger domain-containing protein 1	CAATTTGCTTGCCCGCTTTG	ATGTTGCCCCACAATCGTTG	142	96.1
LOC_Os03g13140	Non-symbiotic hemoglobin 2-1	AGGATTCCGCCAATATTGCC	TCTTGAGCTTGGGGTTCTTCTC	126	94.5
LOC_Os06g51084	1,4-alpha-glucan-branching enzyme, chloroplast precursor	TGAAGATGTTTCGGGCATGC	ACCATTTGCGGTCCTCTTTG	134	100.3
LOC_Os05g46480	Late embryogenesis abundant protein, group 3	AGTGAGCAGGTGAAGAGCAC	TGGCAGAGGTGTCCTTGTTG	115	110.4
LOC_Os03g19427	Nicotianamine synthase	TGAGCAAGCTGGAGTACGAC	GGTCGTAGTTGTCGAACACC	150	83.3
LOC_Os04g38410	Chlorophyll A-B binding protein	ACTTCTTCAACCCGGACTCG	TGGCGTTGGAGAAGTTCTCG	78	100
LOC_Os02g56460	Dehydrogenase	AAGATGATGGCGGAGATGACG	TGGTTGGTGCTGAAGTTGAG	125	101
LOC_Os12g26380	Dirigent	TGATTGTCCCCAGCAATGTG	TTGCGGTTTGGATGGTTTGC	81	93.8
LOC_Os11g43900	*TPH* (normaliser)	CATTGGTGCCAACCCATC	AAGGAGGTTGCTCCTGAAGA	113	100.3

Table 8 List of genes and gene specific primers used for qPCR based validation of root microarray results.

The gene loci corresponds to Osa1 (MSU v7) locus obtained from ricechip.org. The primers were designed using Primer 3 web interface with qPCR suite and checked using nBlast for homology to other known ESTs. Primer efficiencies were determined by producing standard curves for each primer pair at varying cDNA concentration using MxPro Mx3005p software from Stratagene.

3.3. Results

3.3.1. RNA quality control

The bioanalyser generates an electropherogram and a gel like image, along with a RIN value (RNA Integrity Number) for individual RNA samples analysed. Figure 3-3 shows a typical electropherogram obtained for intact leaf and root samples. Both the electropherograms show a level baseline indicating absence of RNA degradation. There are two peaks representing 18S ribosomal RNA and 28S ribosomal RNA in each sample, in the ratio of 1:2. There are three extra peaks visible in the leaf electropherogram that correspond to the chloroplast RNA. The profile indicates that the RNA is intact and suitable for use in microarray analysis. Each of the 24 samples exhibited a similar result. The RNA ladder was used for quantification.

3.3.2. Quality control of the microarray

The normalised signal intensities for each replicate array were aligned with every other replicate and their correlation represented in a heat-map (Figure 3-4). Figure 3-4A shows correlation for leaf arrays for all three treatments and control. The replicate one and replicate two for each treatment show a correlation coefficient of more than 0.9, whereas replicate 3 showed a value lower than 0.9 for each treatment. The control plants bear the closest correlation to the nematode stressed plants and the drought stressed plants bear the closest correlation to the dual stressed plants. Figure 3-4B shows the correlation for root arrays for all three treatments and controls. The replicates of each treatment correlate well with each other with a correlation coefficient of more than 0.9. Following the pattern seen in the leaves the control plants bear the closest correlation to the nematode stressed plants and the drought stressed plants bear the closest correlation to the dual stressed plants.

The normalised probe intensities for probes with 20-100 % intensity values for leaf and root arrays are represented in the box plots in Figure 3-5A and Figure 3-5B respectively. The spread of signal values in the leaf arrays is similar to the one in roots but more probe identities qualified as being significantly different above the cut-off in roots than in leaves.

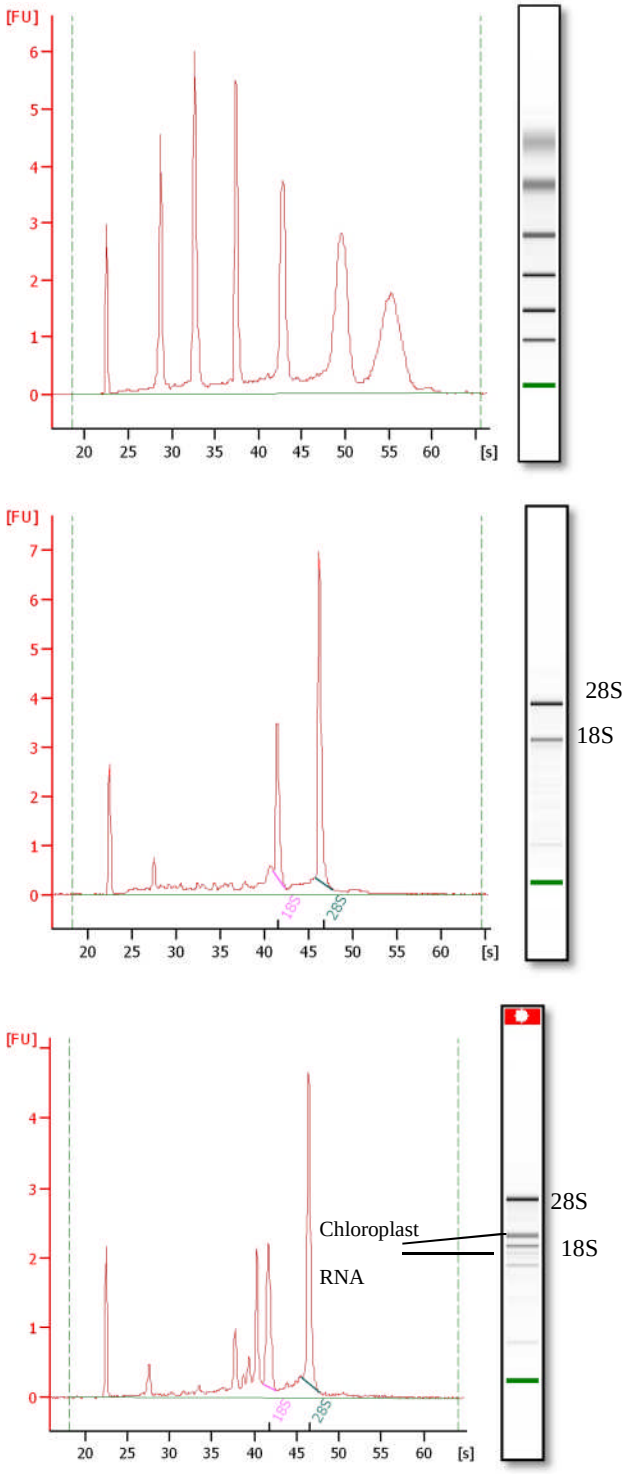


Figure 3-3 Representative electropherograms of leaf and root RNA samples

The Agilent bioanalyser was used to assess the quality of each RNA sample before use in microarray analysis. The horizontal axis represents time and the vertical axis represents fluorescence.

A. The RNA ladder.
B. An electropherogram from a root RNA sample. The RNA is good quality as a flat baseline can be observed and there is a good 1:2 ratio between the two large rRNA peaks.
C. RNA from a leaf sample, in which the 3 extra chloroplast RNA peaks can be observed.

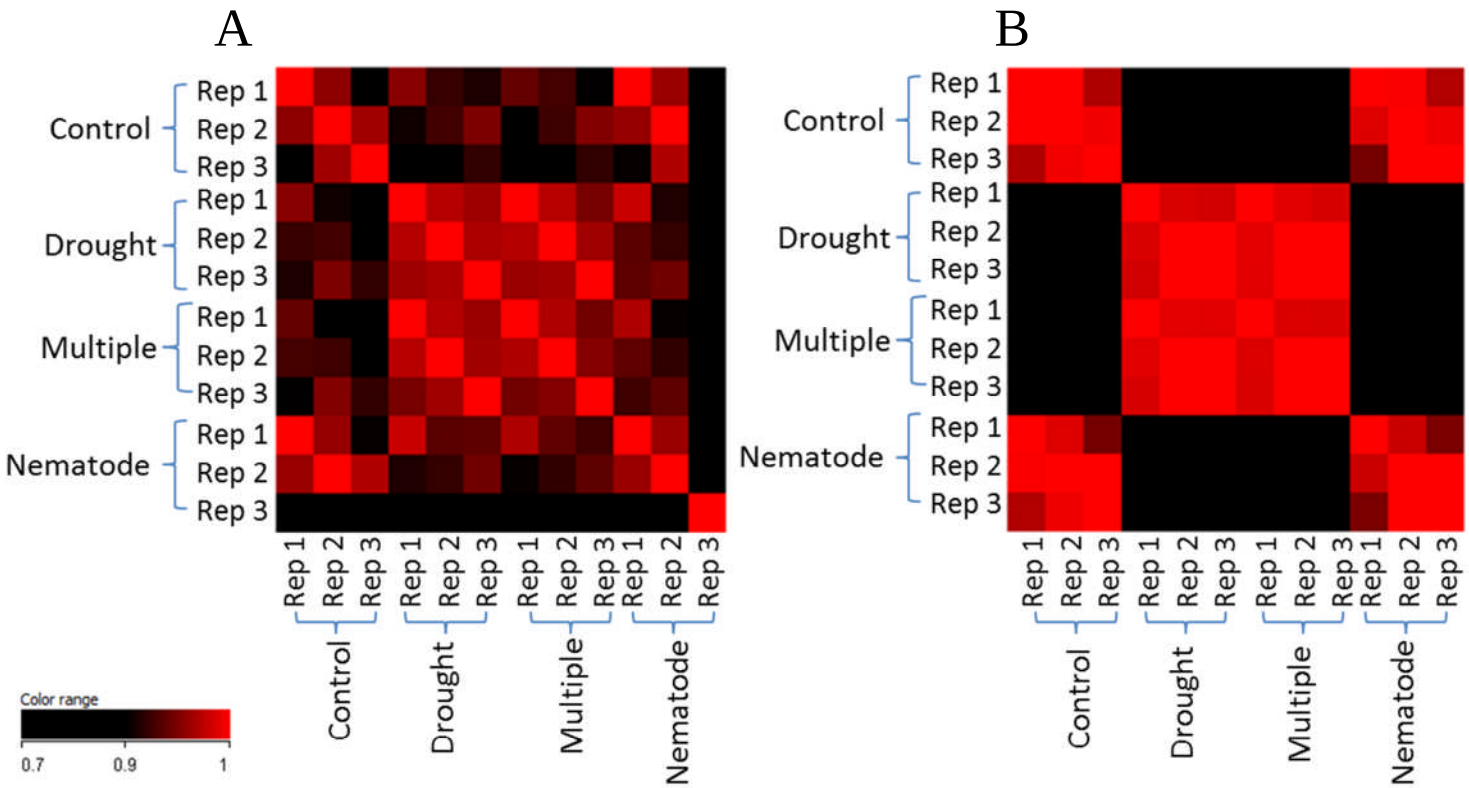
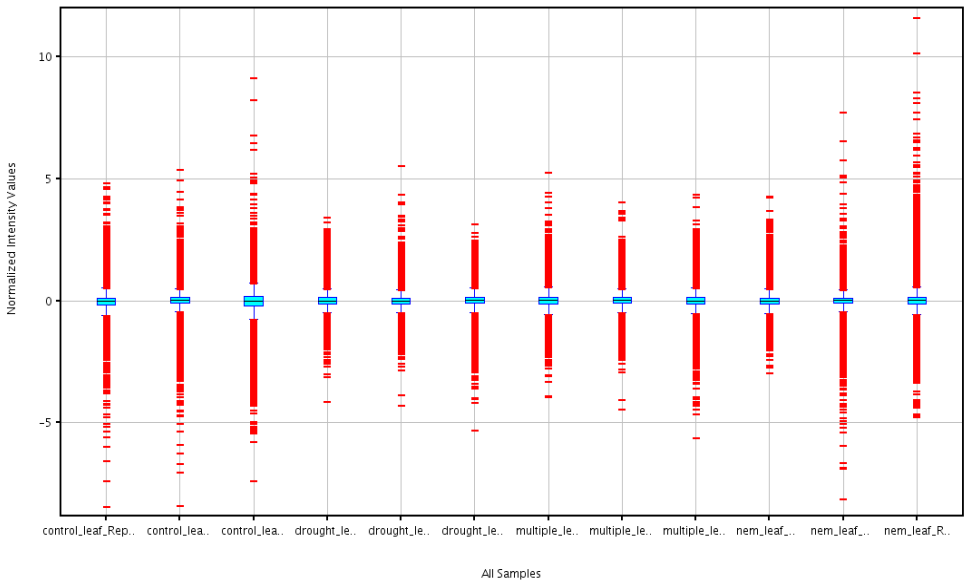


Figure 3-4 Heat map showing correlation between arrays from different treatments

A. Correlation between hybridised probe intensity of three replicates (Rep1, Rep2 and Rep 3) of each stress treatment type (control, drought, multiple and nematode) in leaves.

B. Correlation between hybridised probe intensity of three replicates (Rep1, Rep2 and Rep 3) of each stress treatment type (control, drought, multiple and nematode) in roots.

A



B

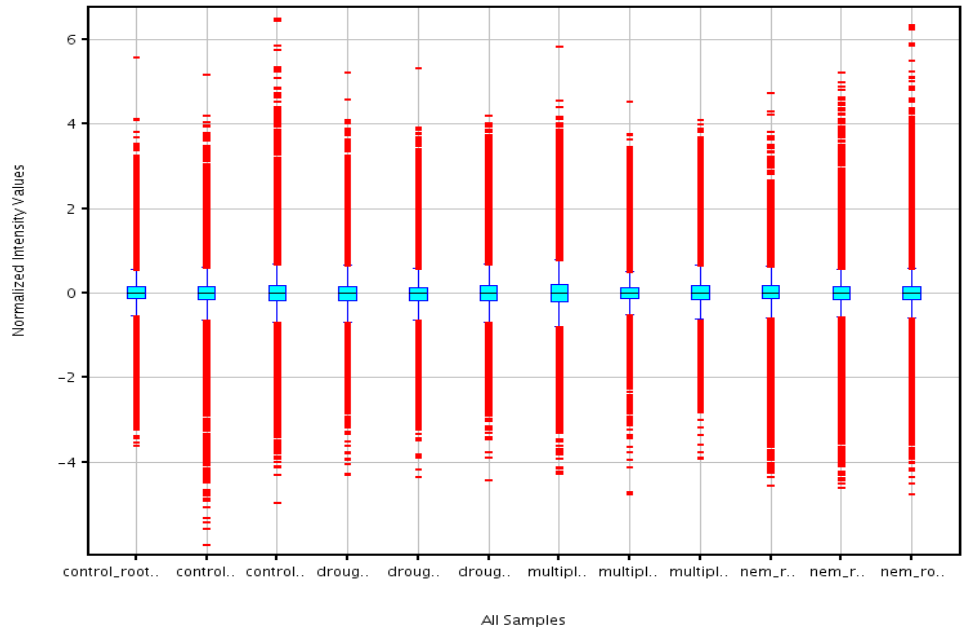


Figure 3-5 Box plot with the normalised probe intensities after PLIER probe summarisation and baseline transformation against median of all files

A. Normalised probe intensities for microarray replicates of leaf tissue under the three treatments and control following the PLIER 16 probe summarisation, quantile normalisation and baseline correction to median of all files.

B. Normalised probe intensities for microarray replicates of root tissue under the three treatments and control following the PLIER 16 probe summarisation, quantile normalisation and baseline correction to median of all files.

3.3.3. Identification of differentially expressed genes

Using Agilent's GeneSpring Gx 12.1 software a one way ANOVA followed by a SNK post-hoc test, applying the Benjamini-Hochberg correction was performed to compare the probe intensities in each treatment with every other treatment. Altogether, 736 genes in leaves and 12,375 genes in roots showed altered expression under one or more of the stress treatments at a significance value of $p \leq 0.05$ (Table 9). The significant probe sets were further filtered on magnitude of the expression change, with a two-fold expression cut-off used to define the list of differentially expressed genes for analysis.

Differentially expressed genes in leaves

After normalisation and baseline correction 53,103 probe sets out of 57,381 had normalised probe intensity higher than 20 %. Following the one way ANOVA with Benjamini-Hochberg multiple testing correction 763 probe sets showed significantly altered expression ($p \leq 0.05$). These 763 probe identities were subjected to a Student-Newman-Keuls post hoc test. The number of genes differentially expressed between different conditions is summarised in Table 9 and Figure 3.6.

More genes were induced than repressed in leaves in response to each of the three stresses. A large proportion of the differentially expressed genes was common between drought and simultaneous multiple stress treatment (362 up-regulated and 105 down-regulated). Fourteen genes were up-regulated and no genes were down–regulated in all three conditions with ≥ two-fold expression change in root and leaf tissue. These up-regulated genes included members of stress responsive families like protein phosphatase 2C, late embryogenesis abundant protein and low temperature and salt responsive proteins (Table 10).

Treatments compared	Tissue	Number of genes differentially regulated ($p \geq 0.05$)
Drought / Control	Leaf	758
Nematode / Control	Leaf	87
Multiple / Control	Leaf	756
Multiple / drought	Leaf	18
Drought / Control	Root	11,776
Nematode / Control	Root	852
Multiple / Control	Root	11,727
Multiple / Drought	Root	789

Table 9 Differentially expressed genes in the stress treatments

Genes differentially expressed in leaves and roots of plants under drought, nematode and simultaneous multiple stress identified by performing a one way ANOVA with Benjamini-Hochberg multiple testing correction followed by a SNK post-hoc test at $p \leq 0.05$.

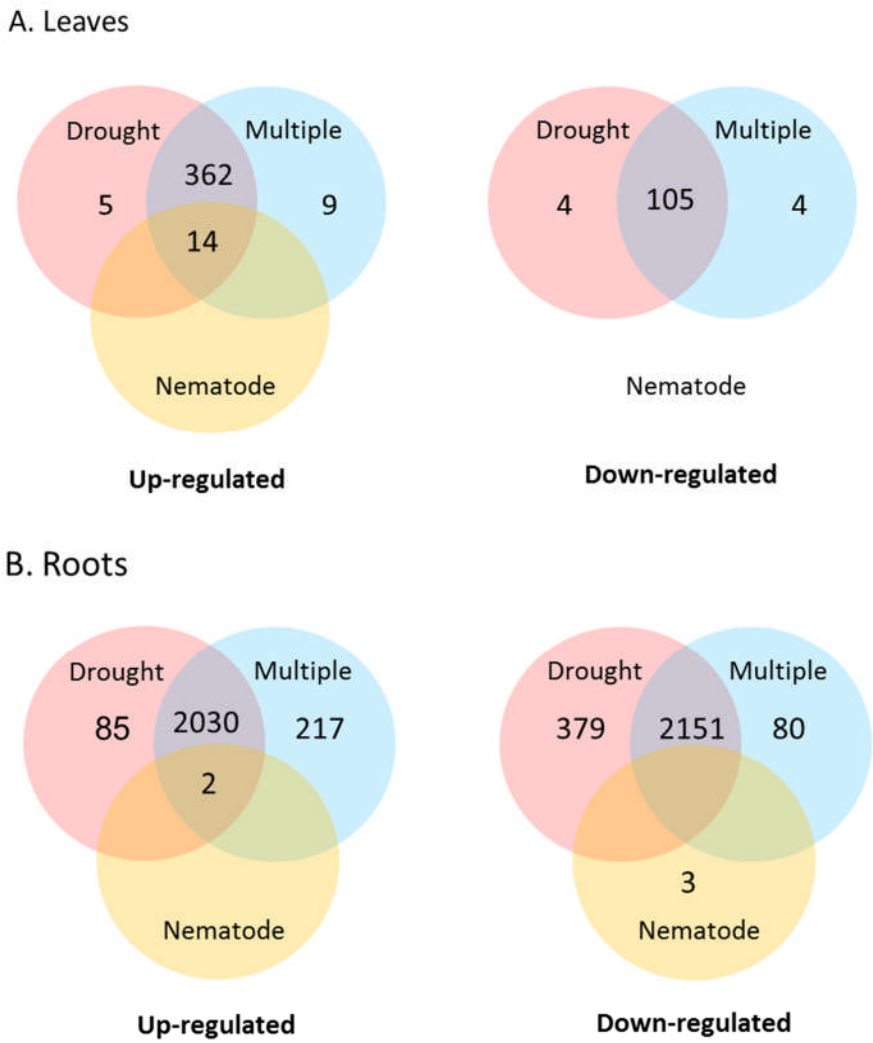


Figure 3-6 Venn diagrams representing the overlap between the genes differently regulated in the three treatments

A. The genes differentially regulated by a 2-fold expression change significant at $p \leq 0.05$ between the three stress treatments in the leaf tissue.

B. The genes differentially regulated by a 2-fold expression change significant at $p \leq 0.05$ between the three stress treatments in the roots.

Affymetrix probe id	Gene locus	Gene annotation	Regulation	Tissue
Os.10933.1.S1_at	**LOC_Os01g50910**	Late embryogenesis abundant protein, group 3	Up	Leaves
Os.11491.1.S1_at	**LOC_Os03g19290**	Mitochondrial import inner membrane translocase subunit Tim17	Up	Leaves
Os.12186.1.S1_at	**LOC_Os03g21040**	Stress responsive protein	Up	Leaves
Os.12415.1.S1_at	**LOC_Os09g02180**	Expressed protein	Up	Leaves
Os.15938.1.S1_at	**LOC_Os03g17790**	OsRCI2-5-Putative low temperature and salt responsive protein	Up	Leaves
Os.34372.1.S1_at	**LOC_Os06g48300**	Protein phosphatase 2C	Up	Leaves
Os.50134.1.S1_at	**LOC_Os05g49730**	Protein phosphatase 2C	Up	Leaves
Os.52036.1.S1_at	**LOC_Os05g31020**	Eukaryotic peptide chain release factor subunit 1-1	Up	Leaves
Os.54997.1.S1_at	**LOC_Os03g51350**	Expressed protein	Up	Leaves
Os.8965.1.S1_at	**LOC_Os01g40280**	Integral membrane protein	Up	Leaves
OsAffx.14523.1.S1_s_at	**LOC_Os05g03130**	OsRCI2-7-Putative low temperature and salt responsive protein	Up	Leaves
OsAffx.18737.1.S1_at	**LOC_Os11g07911**	Expressed protein	Up	Leaves
OsAffx.5702.1.S1_s_at	**LOC_Os08g04560**	Decarboxylase	Up	Leaves
OsAffx.19837.1.S1_at	**LOC_Os12g26380**	Dirigent, putative, expressed	Up	Roots
OsAffx.30997.1.S1_at	**LOC_Os11g16250**	Expressed protein	Up	Roots

Table 10 Genes differentially expressed in leaves or roots in response to all treatments-drought, nematode and simultaneous multiple stresses.

Fifteen genes were differentially expressed in response to drought, nematode and simultaneous multiple stress. Thirteen genes were induced in leaves and two were induced in roots (shaded rows). Their expression was significantly different from the mean of the control with a corrected $p \leq 0.05$, after using Benjamini-Hochberg false discovery rate (FDR) correction.

The number of genes specific to each treatment was very small. Five genes were up-regulated and four down-regulated only in drought stressed plants (Table 11). The list included genes with diverse roles and no specific functional class of genes was over-represented. Nematode stress alone resulted in small expression changes in leaves and none of the genes showed a significant two-fold expression change specific to nematode stress. Only 14 genes qualified the two-fold expression change cut-off in nematode stress, all these genes were common between all three stresses (Table 10). The genes with most significant expression change in leaves in response to nematode infection included four lipid transfer protein genes (LTPL), cytochrome P450, protein phosphatases 2C and uncharacterised proteins (Table 12). Only nine genes were up-regulated and four were down-regulated specifically in response to the simultaneous multiple stresses with ≥ two-fold expression change (Table 13). The list included two cytochrome P450s, a LTPL and genes involved in carbohydrate metabolism, for example starch synthase.

Differentially expressed genes in roots

After normalisation and base line correction, 56,325 probe sets out of 57,831 had normalised probe intensity higher than 20 %. Following the one-way ANOVA with Benjamini-Hochberg multiple testing correction 12,375 probe sets showed significantly altered expression ($p \leq 0.05$). The number of probe sets making through the cut-off in roots was sixteen times higher than for leaves. These 12,375 probe IDs were subjected to a Student-Newman-Keuls post hoc test. The number of genes differentially expressed in roots between different conditions is summarised in Table 9.

Much abundant stress responsive transcriptomic changes were seen in the roots than in leaves for all three treatments. There was a significant two-fold expression change in 6.72 % of the total number of genes present on the array. A larger number of genes were repressed in roots in response to all three treatments. Similar to the pattern seen in leaves, the largest number of DEGs were common between drought and simultaneous multiple stressed plants (2,030 up-regulated and 2,151 down-regulated). Eighty-five genes were up-regulated specifically in response to drought stress alone. Six out of the twenty most significantly induced genes are expressed proteins of unknown function. The remaining genes include abiotic stress responsive protein phosphatases 2C and cold acclimation proteins WCOR413 (Table 14). Three hundred and seventy nine genes

were down-regulated in the drought stressed plants including LTPLs, peroxidases and receptor-like kinases (Table 15). In the simultaneous multiple stressed treatment 217 genes were up-regulated; the first twenty genes with the most significant expression changes include transposon proteins, BZIP transcription factors, glutathione S-transferase and proteins of unknown function (Table 16). Eighty genes were down-regulated specifically in response to the multiple stress, the ten genes with the most significant expression change included cytochrome P450, wall-associated kinases, nodulin-like protein and dirigent proteins (Table 17). Similar to the responses observed in the leaves, the magnitude of expression change in nematode specific gene responses was less than two-fold and none of the genes on array showed significant two fold up-regulation specific to nematode stress, only two genes showed two fold down-regulations. The 20 genes with the most significant expression change in response to the nematode stress in roots include signalling molecules such as calmodulin-binding domain protein, Ras-related protein and protein kinase containing proteins along with six uncharacterised proteins (Table 18). Only two genes were found up-regulated with more than two-fold expression change and they were common in all three conditions (Figure 3-6B). The stress response in roots was more specific to each treatment type and only two genes were common between all three stress responses (2 genes up-regulated) (Table 10).

Tissue-specific differential expression of genes

The drought and the simultaneous multiple stressed plants exhibited tissue-specific as well as general stress responses to the experienced stresses. As a response to drought stress, 210 genes were up-regulated and 27 genes were down-regulated in both tissues. The root-specific drought responses involved up-regulation of 1907 genes and down-regulation of 2503 genes. A higher proportion of genes was up-regulated in leaves in response to the drought stress (157 up-regulated and 82 down-regulated) (Figure 3-7A). A similar trend was observed in response to simultaneous multiple stresses. Two hundred and fourteen genes were commonly up-regulated and 28 genes were commonly down-regulated in both the tissues. Root-specific simultaneous multiple stress responses involved up-regulation of 2036 genes and down-regulation of 2203 genes. One hundred and fifty eight genes were up-regulated whereas only eighty one genes were down regulated in leaves as a response to simultaneous multiple stress (Figure 3.8B). Thirteen genes showed antagonistic expression changes in roots and leaves, in response to

drought and simultaneous stress. Of those, seven genes were down-regulated in roots and up-regulated in leaves in response to drought as well as simultaneous stress. Five and six genes were up-regulated in root and down-regulated in leaves, in response to simultaneous and drought stress, respectively (Table 19). Nodulin genes of two different classes were antagonistically expressed in roots and leaves. Two zinc finger protein (B-box and C3H3 type) transcription factors were up-regulated in roots and down-regulated in leaves under both treatments. The bifunctional 3-phosphoadenosine 5-phosphosulfate synthetase was antagonistically regulated only under the multiple stress treatment.

Affymetrix gene chip probe ids	Osa1 gene locus	Gene name	p-value	Fold change
Os.10880.1.S1_at	LOC_Os04g45270	Aspartyl protease family protein	0.0356	2.26
Os.16884.1.S1_at	LOC_Os03g50870	Expressed protein	0.0293	2.04
Os.36236.2.S1_x_at	LOC_Os01g26390	TKL_IRAK_DUF26-lh.1-DUF26 kinases have homology to DUF26 containing loci	0.0350	2.10
OsAffx.13772.1.S1_x_at	LOC_Os04g11470	Retrotransposon protein	0.0211	2.10
OsAffx.24964.2.S1_s_at	LOC_Os01g13050 LOC_Os04g52190	Long cell-linked locus protein, Vacuolar-sorting receptor precursor	0.0338	2.16
Os.20189.1.S1_at	LOC_Os10g42250	Expressed protein	0.04488	-2.02
Os.5406.1.S1_at	LOC_Os03g58040	Glutamate dehydrogenase protein	0.0244	-2.03
Os.9980.1.S1_at	LOC_Os07g46790	4-alpha-glucanotransferase	0.0086	-2.25
OsAffx.23999.1.S1_x_at	LOC_Os01g71320	Hexokinase	0.0047	-2.05

Table 11 Genes differentially expressed in leaves specifically in response to drought stress only.

Genes that showed more than two-fold expression change only in response to drought stress. Five probe IDs corresponding to seven predicted genes were induced and four probe IDs corresponding to four genes were repressed (shaded rows). The fold change is a mean of three biological replicates, significantly different from the mean of the control with a corrected $p \leq 0.05$, after using Benjamini-Hochberg FDR correction.

Affymetrix probe identities	Gene locus	Gene name	p-value	Fold change
Os.8017.1.S1_at	LOC_Os03g55850	Cold acclimation protein WCOR413	0.0021	1.29
Os.24577.1.S1_at	LOC_Os12g39320	DUF221 domain containing protein	0.0021	1.19
Os.24698.2.S1_x_at	LOC_Os11g37260	SEY1	0.0022	1.28
Os.11611.1.S1_at	LOC_Os02g13350	Hydrolase, NUDIX family, domain containing protein	0.0022	-1.26
OsAffx.25732.1.S1_at	LOC_Os03g56170	Expressed protein	0.0034	1.49
Os.8629.1.S1_x_at	LOC_Os12g02320	LTPL12 - Protease inhibitor/seed storage/LTP family protein precursor	0.0040	1.11
Os.18381.1.S1_at	LOC_Os04g19740	Transketolase	0.0041	1.47
OsAffx.14523.1.S1_s_at	LOC_Os05g03130	OsRCI2-7 - Putative low temperature and salt responsive protein	0.0043	2.41
Os.18257.1.S1_at	LOC_Os07g10840	Uncharacterized glycosyltransferase	0.0047	1.58
Os.9342.1.S1_at	LOC_Os07g43290	LTPL56 - Protease inhibitor/seed storage/LTP family protein precursor	0.0047	1.41
Os.9022.1.S1_at	LOC_Os09g15670	Protein phosphatase 2C	0.0050	1.88
Os.30324.2.S1_x_at	LOC_Os01g44250	CBS domain containing membrane protein	0.0050	1.32
Os.32734.1.S1_at	LOC_Os08g32600	STE_MEKK_ste11_MAP3K.21 - STE kinases include homologs to sterile 7 sterile 11 and sterile 20 from yeast	0.0059	1.16
Os.7802.1.S1_at	LOC_Os04g55800	Sulfate transporter	0.0066	-1.20
Os.20810.3.S1_x_at	LOC_Os01g56300	Expressed protein	0.0069	1.28
	LOC_Os02g31220	Expressed protein		
Os.57455.1.S1_x_at	LOC_Os11g02330	LTPL22 - Protease inhibitor/seed storage/LTP family protein precursor	0.0069	1.35
	LOC_Os12g02290	LTPL23 - Protease inhibitor/seed storage/LTP family protein precursor		
Os.15637.1.S1_s_at	LOC_Os03g14420	Cytochrome P450	0.0069	1.19
OsAffx.24138.1.S1_s_at	LOC_Os02g06720	WD domain containing protein	0.0073	1.67
Os.11491.1.S1_at	LOC_Os03g19290	Mitochondrial import inner membrane translocase subunit Tim17	0.0074	2.41
Os.12415.1.S1_at	LOC_Os09g02180	Expressed protein	0.0075	2.83

Table 12 Genes that were most significantly differentially expressed in leaves as a response to nematode infection.

Twenty genes that were most significantly differentially expressed in leaves in response to nematode stress were taken and the ones with an assigned gene locus are presented here, in order of decreasing significance. The fold change is a mean of three biological replicates, significantly different from the mean of the control with a corrected p value of ≤ 0.05, after using Benjamini-Hochberg FDR correction.

Affymetrix gene chip probe ids	Osa1 gene locus	Gene name	p-value	Fold change
Os.10615.1.S1_x_at	LOC_Os12g02310	LTPL11-Protease inhibitor/seed storage/LTP family protein	0.0472	2.08
Os.15637.1.S1_s_at	LOC_Os03g14420	Cytochrome P450	0.0069	2.08
Os.27861.1.S2_a_at		No gene assigned	0.0196	2.26
Os.50953.1.S1_at	LOC_Os06g49970	Alpha-amylase precursor	0.0219	2.11
Os.56168.1.S1_at	LOC_Os05g40384	Cytochrome P450	0.0102	2.11
Os.6350.1.S1_at	LOC_Os03g05110	Xyloglucan galactosyltransferase KATAMARI1	0.0162	2.03
Os.7711.1.S1_at	LOC_Os10g18370	Transcriptional regulator	0.0458	2.08
OsAffx.24977.1.S1_x_at	LOC_Os03g05330	HEAT repeat family protein	0.0338	2.08
OsAffx.25768.1.S1_x_at	LOC_Os03g59480	Expressed protein	0.0359	2.35
Os.10240.1.S1_at	LOC_Os01g65680	4,5-DOPA dioxygenase extradiol	0.0359	-2.04
Os.11244.2.S1_x_at	LOC_Os06g04200	Starch synthase	0.0482	-2.05
Os.23290.2.S1_s_at	LOC_Os10g40960	Oxidoreductase, 2OG-Fe oxygenase family protein	0.0074	-2.07
OsAffx.30129.1.S1_at	LOC_Os09g33830	Solute carrier family 35 member F1	0.0351	-2.08

Table 13 Genes differentially expressed in leaves only in response to the simultaneous multiple stresses.

Thirteen genes were differentially expressed in leaves specifically in response to simultaneous drought and nematode stress. Nine were induced and four were repressed (shaded rows). The fold change is a mean of three biological replicates, significantly different from the mean of the control with a corrected p-value of ≤ 0.05, after using Benjamini-Hochberg FDR correction.

Affymetrix gene chip Probe ID	Gene locus	Gene name	p-value	Fold change
OsAffx.12887.2.S1_s_at	LOC_Os03g16920	DnaK family protein	1.05×10^{-6}	77.31
OsAffx.2045.2.S1_at	LOC_Os01g20850	Expressed protein	1.05×10^{-6}	7.781
Os.178.1.S1_a_at	LOC_Os08g25734	Glucose-1-phosphate adenylyltransferase large subunit	2.58×10^{-6}	3.65
Os.51775.1.S1_x_at	LOC_Os12g05210	Expressed protein	2.76×10^{-6}	69.09
Os.34372.1.S1_at	LOC_Os06g48300	Protein phosphatase 2C	2.76×10^{-6}	57.16
Os.55461.1.S1_at	LOC_Os03g26490	Expressed protein	$2.82x\ 10^{-6}$	104.37
Os.39552.1.A1_s_at	LOC_Os06g48300	Protein phosphatase 2C	2.82×10^{-6}	75.67
Os.46582.2.S1_x_at	LOC_Os10g36180	Expressed protein	2.82×10^{-6}	64.96
Os.36307.1.S1_at	LOC_Os04g35490	Expressed protein	2.82×10^{-6}	48.77
Os.8897.1.S1_at	LOC_Os06g44140	Transmembrane 9 superfamily member	2.82×10^{-6}	37.62
Os.23224.1.S1_s_at	LOC_Os06g32550	THION14-Plant thionin family protein	2.82×10^{-6}	16.04
OsAffx.12986.1.S1_at	LOC_Os03g22200	Nodulin MtN3 family protein	2.82×10^{-6}	13.20
Os.8017.1.S1_at	LOC_Os03g55850	Cold acclimation protein WCOR413	2.82×10^{-6}	7.21
Os.27578.1.S1_at	LOC_Os01g33420	Glycosyl hydrolase family protein 27	2.82×10^{-6}	3.51
Os.7835.1.S1_a_at	LOC_Os08g09250	Glyoxalase family protein	2.82×10^{-6}	2.21
Os.8195.1.S1_at	LOC_Os02g50700.1	-	2.82×10^{-6}	6.65
Os.27578.1.S1_at	LOC_Os01g33420	Glycosyl hydrolase family protein 27	2.82×10^{-6}	3.51
Os.7835.1.S1_a_at	LOC_Os08g09250	Glyoxalase family protein	2.82×10^{-6}	2.21
Os.39596.1.A1_x_at	LOC_Os07g16610.1	-	2.98×10^{-6}	17.04
Os.54997.1.S1_at	LOC_Os03g51350	Expressed protein	3.01×10^{-6}	105.32

Table 14 Genes most significantly induced in roots specifically in response to drought stress

First 20 probe identities that were most significantly induced in roots in response to only drought stress are presented here, in decreasing order of significance. The fold change is a mean of three biological replicates, significantly different from the mean of the control with a corrected $p \leq 0.05$, after using Benjamini-Hochberg FDR correction.

Affymetrix gene chip probe ids	Osa1 gene locus	Gene name	p-value	Fold change
OsAffx.30623.1.S1_at	LOC_Os10g31180	Hypothetical protein	1.05×10^{-6}	-6.30
Os.7694.1.S1_at	LOC_Os04g41229	Helix-loop-helix DNA-binding domain containing protein, expressed	2.82×10^{-6}	-29.75
Os.27793.1.S1_at	LOC_Os02g14430	Peroxidase precursor	3.01×10^{-6}	-15.77
Os.4194.1.S1_at	LOC_Os10g40430	LTPL139-Protease inhibitor/seed storage/LTP family protein precursor	3.95×10^{-6}	-77.68
Os.8815.1.S1_at	LOC_Os11g10510	Dehydrogenase	3.95×10^{-6}	-11.11
OsAffx.19356.1.S1_at	LOC_Os11g43770	Leucine Rich Repeat family protein	3.95×10^{-6}	-5.31
OsAffx.26728.1.S1_at	LOC_Os05g04470	Peroxidase precursor	4.25×10^{-6}	-6.97
Os.12092.1.S1_at	LOC_Os09g25810	Nodulin	4.97×10^{-6}	-3.31
Os.17190.1.A1_s_at	LOC_Os11g36150	Receptor-like protein kinase 2 precursor	5.11×10^{-6}	-5.47
Os.56910.1.S1_s_at	LOC_Os07g48390	Proline-rich protein	5.18×10^{-6}	-5.31
OsAffx.15306.1.S1_s_at	LOC_Os06g08210	-	5.25×10^{-6}	
Os.4159.1.S1_at	LOC_Os01g51570	Glycosyl hydrolases family 17	5.69×10^{-6}	-28.47
OsAffx.10896.1.S1_at	LOC_Os01g03640	Multicopper oxidase domain containing protein	6.18×10^{-6}	-13.10
Os.8256.1.S1_at	LOC_Os12g38760	Nucleotide pyrophosphatase/phosphodiesterase	6.69×10^{-6}	-5.17
Os.8416.1.S1_at	LOC_Os03g57530	DUF593 domain containing protein	6.69×10^{-6}	-2.00
OsAffx.23371.1.S1_x_at	LOC_Os01g24420	Expressed protein	8.06×10^{-6}	-5.65
Os.50127.1.S1_at	LOC_Os04g30030	Cystiene-rich receptor-like protein kinase 12 precursor	8.79 x 10-6	-7.39
Os.49381.1.S1_at	LOC_Os08g26820	Plant protein of unknown function domain containing protein,	8.79×10^{-6}	-7.12
Os.42024.1.S1_at	LOC_Os01g56420	Ctr copper transporter family protein	1.01 x 10-5	-12.12
OsAffx.30934.1.S1_at	LOC_Os11g11650	Expressed protein	1.08 x 10-5	-8.24

Table 15 Genes that were most significantly repressed in roots specifically in response to drought stress

First 20 probe identities that were most significantly repressed in roots in response to only drought stress were taken and the ones with an assigned gene locus are presented here, in order of decreasing significance. The fold change is a mean of three biological replicates, significantly different from the mean of the control with a corrected $p \leq 0.05$, after using Benjamini-Hochberg FDR correction.

Affymetrix probe ids	Gene locus	Gene name	P value	Fold change
Os.12101.2.A1_a_at	LOC_Os03g31839	Transposon protein	7.74×10^{-5}	2.04
Os.1741.3.A1_at	LOC_Os01g26130	Expressed protein	1.90×10^{-4}	2.14
Os.49514.1.S1_at	LOC_Os02g16680	BZIP transcription factor domain containing protein,	1.90×10^{-4}	2.06
Os.30790.1.S1_at	LOC_Os01g27480	Glutathione S-transferase	2.46×10^{-4}	2.13
OsAffx.13462.1.S1_at	LOC_Os03g51990	ACT domain containing protein	2.52×10^{-4}	2.04
Os.10616.1.S1_a_at	LOC_Os01g73970	Lysine ketoglutarate reductase trans-splicing related 1	2.54×10^{-4}	2.04
Os.7059.2.S1_x_at	LOC_Os09g04890	Histone-lysine N-methyltransferase, H3 lysine-4 specific ATX1	3.14×10^{-4}	2.07
OsAffx.12132.1.S1_at	LOC_Os02g20450	Hypothetical protein	3.37×10^{-4}	2.36
Os.50588.1.S1_at	LOC_Os11g34370	Phospholipase, patatin family	3.38×10^{-4}	2.07
Os.53735.1.S1_at	LOC_Os06g11660	Phosphate-induced protein 1 conserved region domain containing protein	3.52×10^{-4}	2.14
Os.35785.1.S1_x_at	LOC_Os01g35160	TKL_IRAK_DUF26-lh.4-DUF26 kinases have homology to DUF26 containing loci	3.83×10^{-4}	2.04
Os.27667.1.S1_at	LOC_Os10g35200	Katanin p80 WD40 repeat-containing subunit B1 homolog 1	4.02×10^{-4}	2.09
Os.26996.1.S1_at	LOC_Os08g15080	Proline rich protein 3, putative	4.31×10^{-4}	2.09
Os.50641.2.S1_a_at	LOC_Os08g33488	OsMADS23-MADS-box family gene with MIKCc type-box	4.43×10^{-4}	2.17
OsAffx.13658.1.S1_at	LOC_Os04g04210	Expressed protein	4.43×10^{-4}	2.16
Os.1606.2.S1_a_at	LOC_Os01g31680	Uncharacterized protein sll0194	5.02×10^{-4}	2.02
OsAffx.3772.1.S1_at	LOC_Os04g12050	Retrotransposon	5.06×10^{-4}	2.10

Table 16 Genes that were most significantly induced in root only in response to simultaneous multiple stresses.

The probe identities that were most significantly induced in response to only simultaneous multiple stress in roots are presented in order of decreasing significance. The fold change is a mean of three biological replicates, significantly different from the mean of the control with a corrected p-value of ≤ 0.05, after using Benjamini-Hochberg FDR correction**.**

Affymterix probe ids	Gene locus	Gene names	p-value	Fold change
Os.5179.1.A1_at	LOC_Os08g36310	Cytochrome P450,	1.52×10^{-5}	-13.52
Os.11417.1.S1_at	LOC_Os02g26770	Cytochrome P450	1.55×10^{-5}	-13.19
Os.49796.1.S1_at	LOC_Os04g51030	Wall-associated kinase 1,	1.56×10^{-5}	-3.32
Os.56657.1.S1_at	LOC_Os06g51190	Lysine ketoglutarate reductase trans-splicing related 1	1.59×10^{-5}	-2.12
OsAffx.24724.1.S1_x_at	LOC_Os02g44599	Expressed protein	1.665×10^{-5}	-3.92
Os.4655.1.S1_at	LOC_Os01g65880	Nodulin MtN3 family protein	1.66×10^{-5}	-11.54
Os.49091.1.S1_at	LOC_Os05g05270	Sucrose-phosphatase	2.31×10^{-5}	-3.10
Os.9709.1.A1_at	LOC_Os08g06380	CSLF6-cellulose synthase-like family F	2.34×10^{-5}	-6.18
Os.50505.2.S1_at	LOC_Os04g42650	Plant protein of unknown function domain	2.35×10^{-5}	-2.09
Os.14539.1.S1_at	LOC_Os07g01600	Dirigent	1.55×10^{-5}	-12.29

Table 17 Genes most significantly repressed in roots in response to only simultaneous multiple stresses

The probe identities that were most significantly repressed in roots in response to only simultaneous multiple stress presented in order of decreasing significance. The fold change is a mean of three biological replicates, significantly different from the mean of the control with a corrected p value of ≤ 0.05, after using Benjamini-Hochberg FDR correction.

Affymetrix gene chip probe ids	Osa1 gene locus	Gene name	p-value	Fold change
OsAffx.30623.1.S1_at	**LOC_Os01g61720**	IQ calmodulin-binding motif domain containing protein	1.5×10^{-6}	1.17
Os.178.1.S1_a_at	**LOC_Os08g25734**	Glucose-1-phosphate adenylyltransferase large subunit chloroplast precursor	2.5×10^{-6}	1.14
Os.27578.1.S1_at	**LOC_Os01g33420**	Glycosyl hydrolase family protein 27	2.8×10^{-6}	1.18
Os.56657.1.S1_at	**LOC_Os06g51190**	Lysine ketoglutarate reductase trans-splicing related 1	1.59×10^{-5}	1.16
OsAffx.24885.1.S1_at	**LOC_Os02g55540**	F-box/LRR-repeat protein 14	1.89×10^{-5}	-1.16
OsAffx.19837.1.S1_at	**LOC_Os12g26380**	Dirigent	2.18×10^{-5}	-2.41
Os.33755.1.S1_at	**LOC_Os03g05280**	Ras-related protein	1.17×10^{-5}	1.17
Os.46498.1.S1_at	**LOC_Os10g11310**	Expressed protein	2.4×10^{-5}	1.31
Os.16896.1.S1_at	**LOC_Os09g33630**	Protein kinase domain containing protein	3.18×10^{-5}	1.18
Os.24050.1.S1_at	**LOC_Os12g27440**	Expressed protein	3.32×10^{-5}	1.44
Os.19143.1.S1_at	**LOC_Os05g45890**	TRNAHisguanylyltransferase family protein	4.88×10^{-5}	1.11
Os.30589.1.S1_at	**LOC_Os03g40040**	Expressed protein	5.11×10^{-5}	-1.12
Os.49030.1.A1_s_at	**LOC_Os09g20220**	Glutathione S-transferase	5.22×10^{-5}	-1.43
Os.9330.1.S1_x_at	**LOC_Os02g41630**	Phenylalanine ammonia-lyase	6.57×10^{-5}	1.28
OsAffx.26763.1.S1_at	**LOC_Os05g06740**	Expressed protein	6.75×10^{-5}	1.25
Os.16922.1.S1_at	**LOC_Os09g33530**	Expressed protein	8.26×10^{-5}	1.15
Os.27426.1.S1_at	**LOC_Os05g44080**	Transmembrane protein, putative	9.34×10^{-5}	1.17
Os.18983.1.S1_at	**LOC_Os03g52180**	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	9.95×10^{-5}	1.39
Os.52217.1.S1_at	**LOC_Os09g14410**	Expressed protein	1.00×10^{-4}	1.12

Table 18 Genes that were most significantly differentially expressed in roots as a response to nematode infection.

First twenty probe identities that were most significantly differentially expressed in roots in response to nematode stress were taken and the ones with an assigned gene locus are presented here, in order of decreasing significance. The fold change is a mean of three biological replicates, significantly different from the mean of the control with a corrected p-value of ≤ 0.05, after using Benjamini-Hochberg FDR correction.

Figure 3-7. Overlap between the genes differently regulated in the roots and leaves

A. The genes differentially regulated by a 2-fold expression change significant at $p \leq 0.05$ in response to drought stress.

B. The genes differentially regulated by a 2-fold expression change significant at $p \leq 0.05$ in response to simultaneous multiple stress.

Gene Locus	Gene Name	Fold change in drought stress		Fold change in simultaneous stress	
		root	leaf	root	Leaf
LOC_Os09g39430	GDSL-like lipase/acylhydrolase	-3.56	2.24	-2.57	2.35
LOC_Os01g52260	Serine acetyltransferase protein	-3.46	2.49	-3.10	2.47
LOC_Os01g58640	Nucleotide pyrophosphatase/phosphodiesterase,	-3.66	3.75	-3.30	3.58
LOC_Os01g36790	TKL_IRAK_DUF26-lf.2-DUF26 kinases have homology to DUF26 containing loci,	-3.60	3.44	-3.11	3.62
LOC_Os01g42090	Nodulin MtN3 family protein	-5.14	3.40	-4.43	3.33
LOC_Os01g08470	Retrotransposon protein	-2.47	2.70	-2.01	2.83
LOC_Os08g40420	Ternary complex factor MIP1	-2.98	3.8519	-2.91	3.442
LOC_Os12g39110	Zinc finger, C3HC4 type domain containing protein	3.63	-2.39	3.63	-2.244
LOC_Os01g17214	Major facilitator superfamily antiporter	2.77	-3.06	2.97	-3.17
LOC_Os04g02050	Bifunctional 3-phosphoadenosine 5-phosphosulfate synthetase			1.97	-2.44
LOC_Os12g11620	Expressed protein	4.00	-2.30	4.53	-2.93
LOC_Os12g29950	Nodulin, putative	6.72	-3.05	6.41	-3.37
LOC_Os04g45690	B-box zinc finger family protein	3.23	-4.53	3.04	-4.22

Table 19 Genes that have antagonistic expression profiles between roots and leaves in drought and simultaneous stressed plants

Thirteen genes showed antagonistic expression in roots and in leaves under drought and simultaneous multiple stress treatments. Seven genes were repressed in the roots and induced in the leaves (shaded rows) and six genes were repressed in leaves and induced in roots. The fold change is a mean of three biological replicates, significantly different from the mean of the control with a corrected $p \leq 0.05$, after using Benjamini-Hochberg FDR correction.

3.3.4. Validation of microarray results using qPCR

The log2 fold change in expression of transcript determined by qPCR was plotted against the log2 fold change values obtained from microarray analysis and the correlation coefficient was calculated for each treatment. Figure 3-8 shows a scatter plot of the expression values in the leaves for the chosen genes across the three treatments relative to the control. The x-axis represents relative expression as obtained from the microarray and the y-axis shows the relative expression of the same transcript as determined by qPCR. Due to smaller magnitude of changes observed in nematode stressed plants only two points could be obtained. The correlation values for the relative expression determined by microarray and qPCR for genes in drought and simultaneous multiple treatments are 0.92 and 0.99, respectively. Figure 3-9 shows a scatter plot for the expression values for the chosen genes across the three treatments relative to the control in the roots. The magnitude of expression change in nematode stressed roots was low, similar to the leaves and thus limited the number of points obtained, with a correlation value of 0.60. The correlation coefficients for the drought stressed and simultaneous multiple stress treatments for root tissue were 0.90 and 0.91, respectively.

3.3.5. Functional annotation of the differentially expressed genes

Functional annotation of the genes differentially expressed in leaves

The DEGs in drought and simultaneous multiple stressed leaves show similar functional profiles (Figure 3-10 and Figure 3-11). Both of these stresses significantly affected expression of genes involved in cellular nitrogen compound metabolism and macromolecule localisation, specially the lipid transport (red boxes). Both treatments do affect genes involved in the osmotic stress responses and salt stress responses. They also affected carbohydrate metabolism especially starch, sucrose and galactose metabolism. Simultaneous multiple stresses also affected amine metabolic processes, which were not significantly altered in the drought stress responses. Nematode stress significantly altered expression of only the stress responsive genes and unlike the drought and multiple stress treatments, did not have significantly affect the expression of genes belonging to metabolic functional categories.

Functional annotation of the genes differentially expressed in roots

In roots, 193 biological process were significantly affected ($p \leq 0.01$) as a response to drought stress and 206 biological processes were significantly affected ($p \leq 0.01$) as a response to the simultaneous stress. The top ten % of each has been represented in Figures 3-13 and 3-14 Both the treatments showed a very high response to stimuli, which includes response to chemical stimulus, endogenous stimulus, abiotic stimulus and cellular responses. Simultaneous stress also influenced the genes responsive towards general stress and those involved in hormone metabolism and cellular homeostasis.

Nematode stress induced a distinctive pattern of response in roots. It affected cell differentiation, organ development and post embryonic development. None of the genes in the stress responsive family are significantly over represented in the DEG list whereas the related auxin responses are influenced (Figure 3-14).

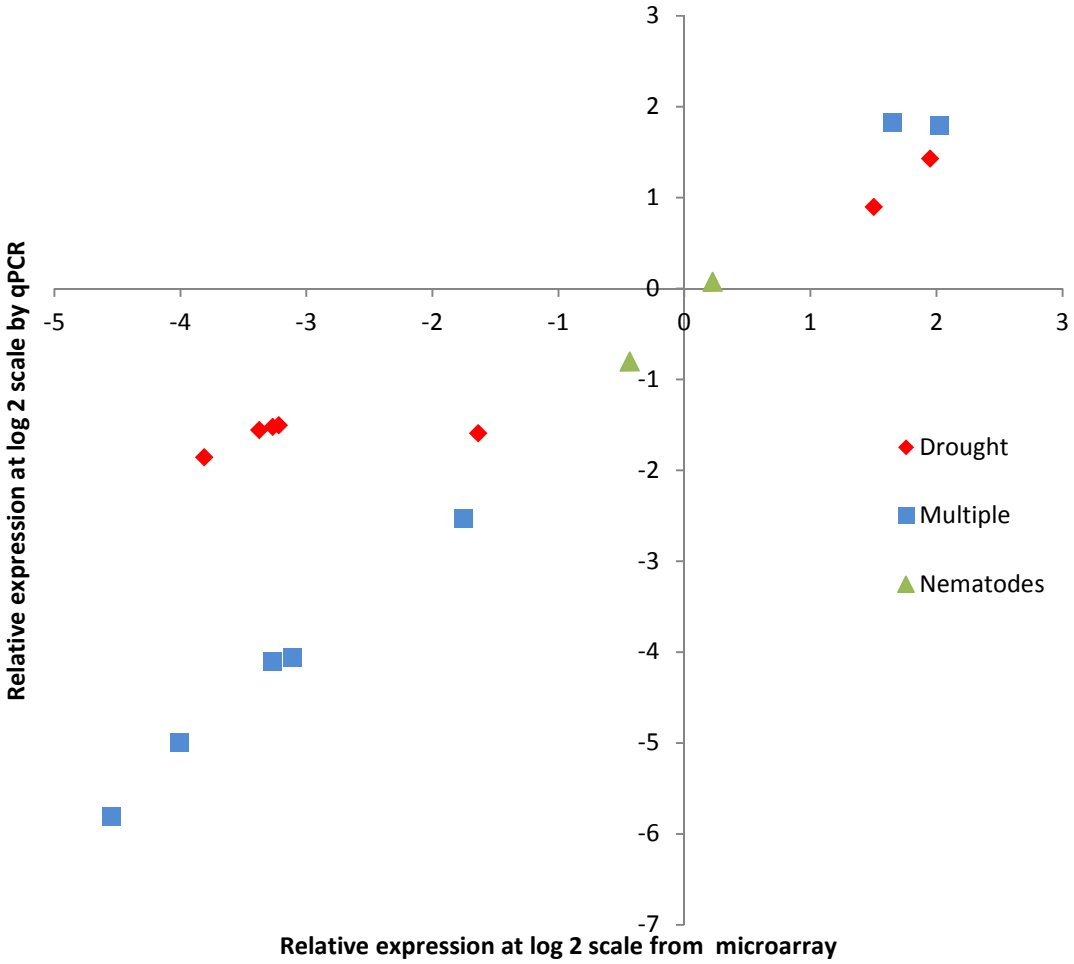


Figure 3-8 Correlation between microarray result and expression analysis using qPCR for leaf samples. Expression of seven randomly selected genes was studied by performing qPCR on RNA extracted from pooled leaf tissue of three biological replicates. These genes had a range of positive and negative relative expression values across the three treatments. The expression was analysed in all three treatments and a strong correlation was observed for drought and multiple stress treatment. (R^2 values: drought 0.927 and multiple 0.9974).

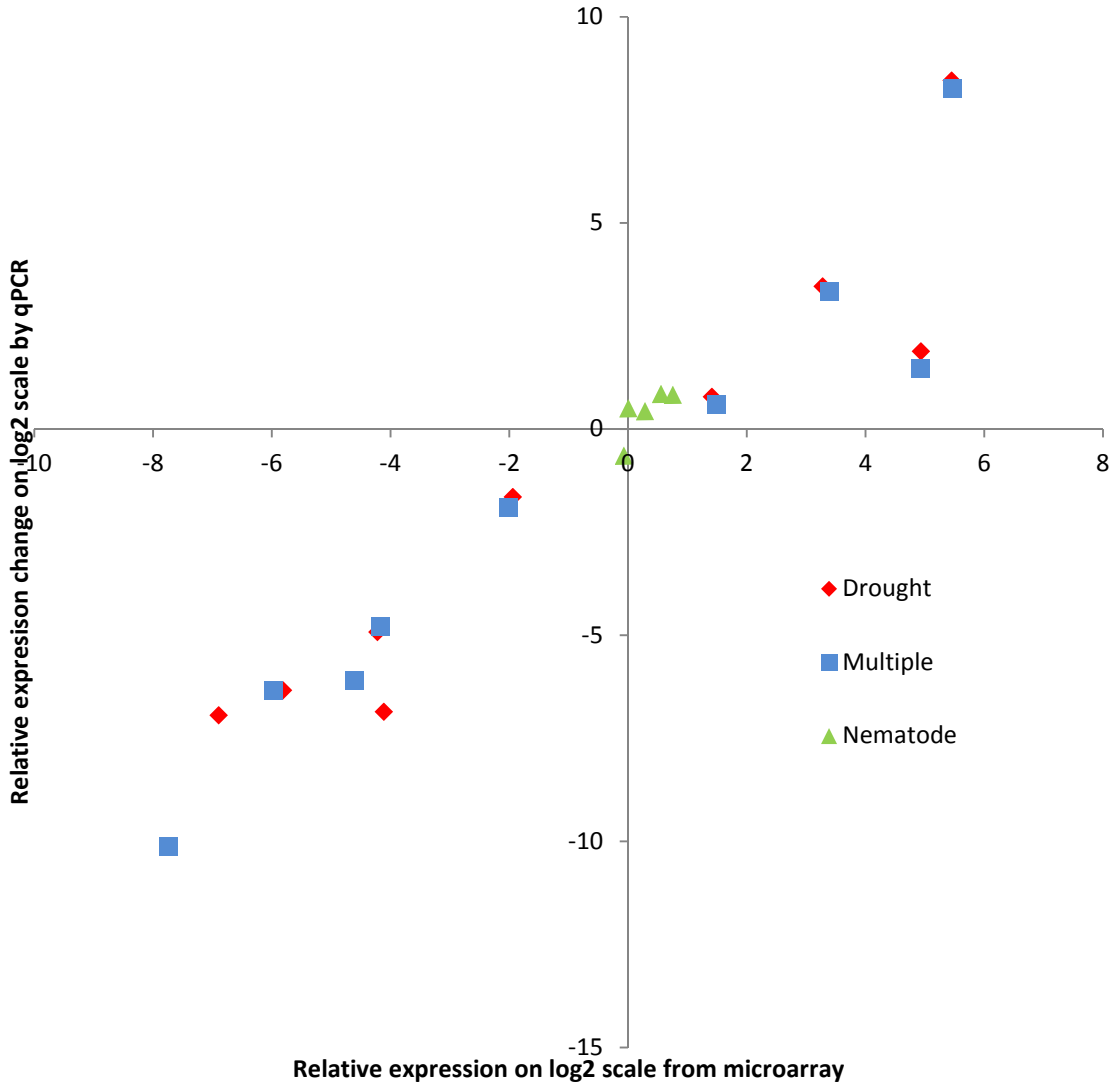


Figure 3-9. Correlation between microarray result and expression analysis using qPCR for root samples. Expression of ten randomly selected genes was studied by performing qPCR on RNA extracted from pooled leaf tissue of three biological replicates. These genes had positive and negative relative expression values across the three treatments. The expression was analysed in all three treatments and a strong correlation was observed for drought and multiple stress treatment. (R^2 values: drought 0.90 and multiple 0.91).

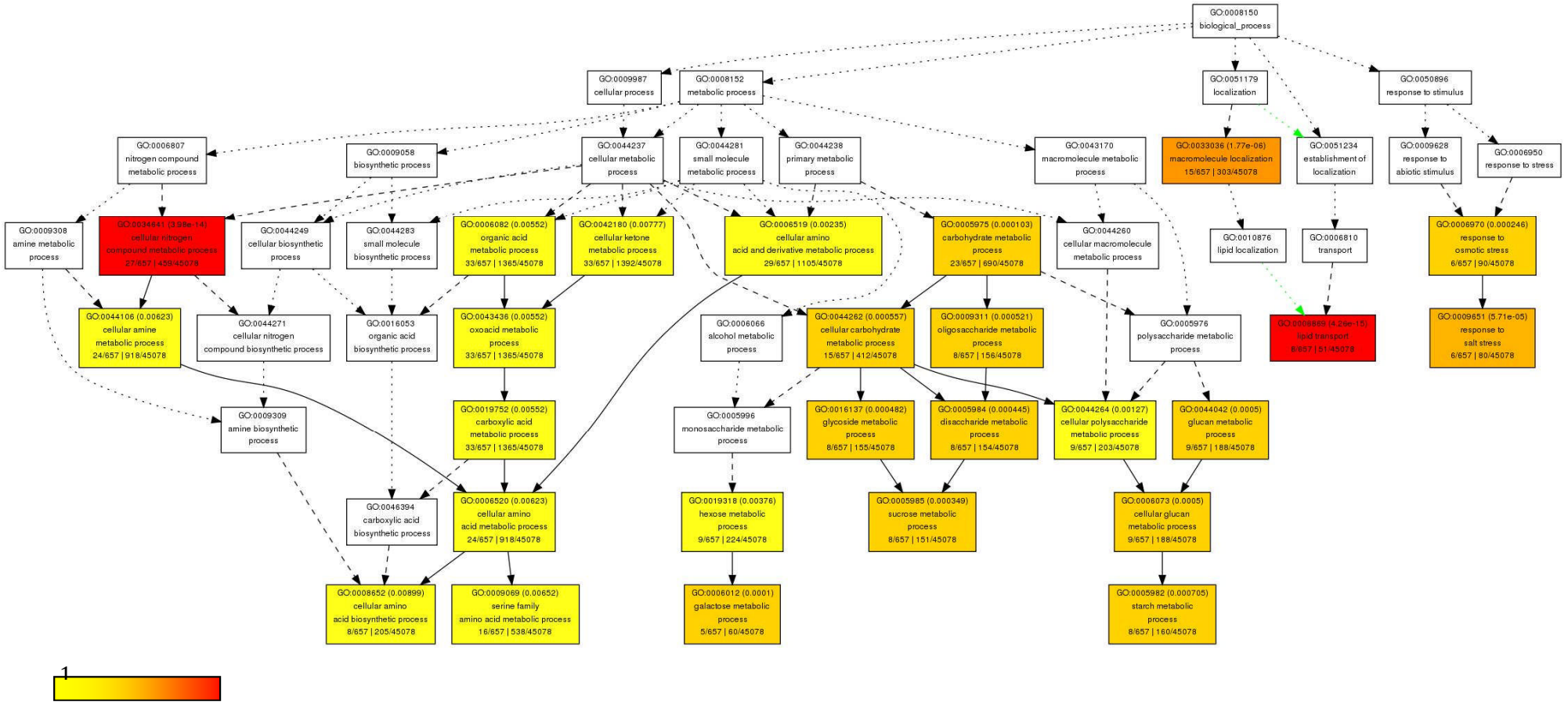


Figure 3-10 Gene ontology enrichment analysis for genes significantly differentially regulated in leaves under drought stress. The number of genes associated with each biological process in the DEGs list was compared to the proportion of genes included on the microarray in the same biological process using singular enrichment analysis by agriGO. Each square represents a GO category followed by significance in the brackets, name of each GO category, ratio of genes in the list and ratio of genes on the microarray belonging to that category. The squares are colour coded for level of significance; yellow is significance level 1 and red is most significant, level 9. The green arrows show negative regulation.

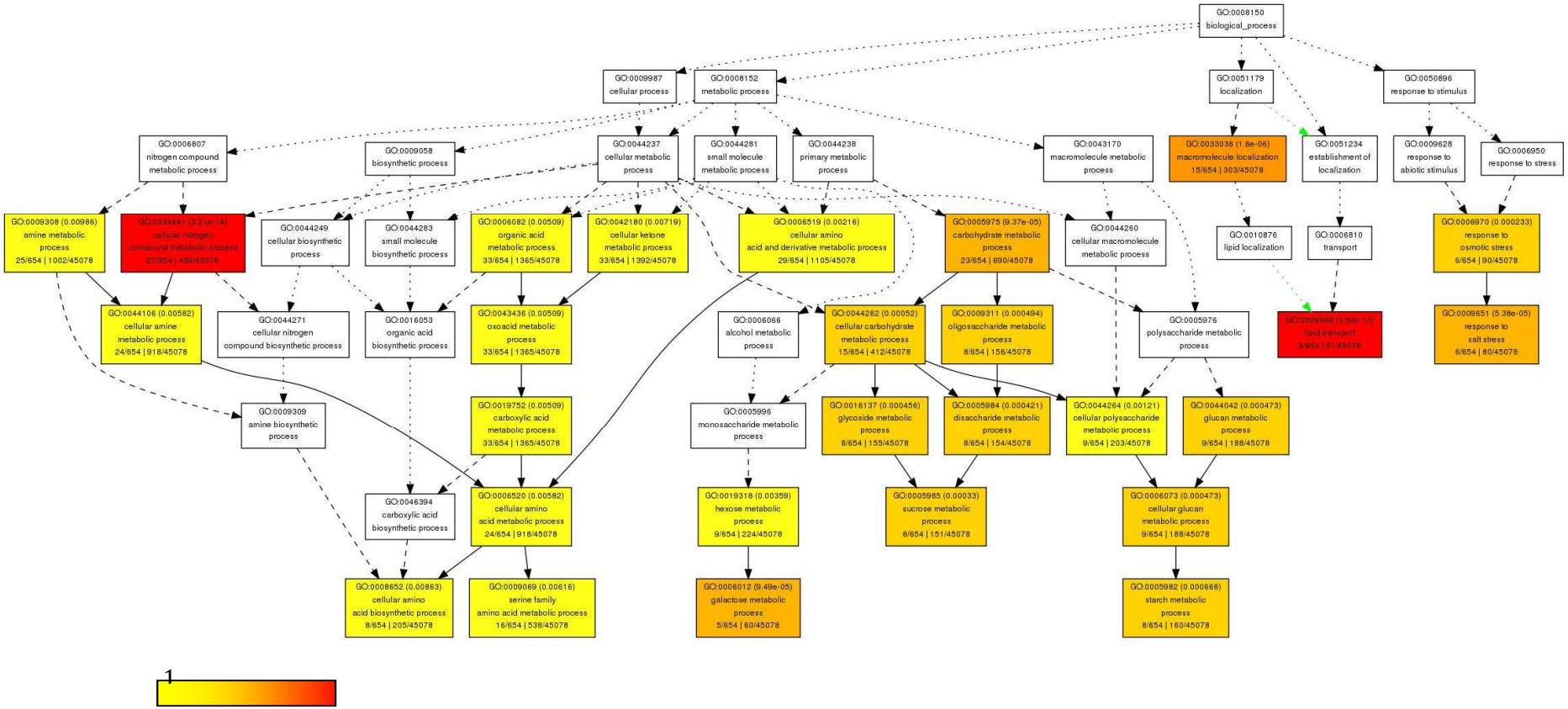


Figure 3-11. Gene ontology enrichment analysis for genes significantly differentially regulated in leaves under simultaneous multiple stress. The number of genes associated with each biological process in the DEGs list was compared to the proportion of genes on the microarray belonging to same biological process using singular enrichment analysis by agriGO. Each square represents a GO category followed by significance in the brackets, name of each GO category, ratio of genes in the list and ratio of genes on the microarray belonging to that category. The squares are colour coded for level of significance; yellow is significance level 1 and red is most significant, level 9.

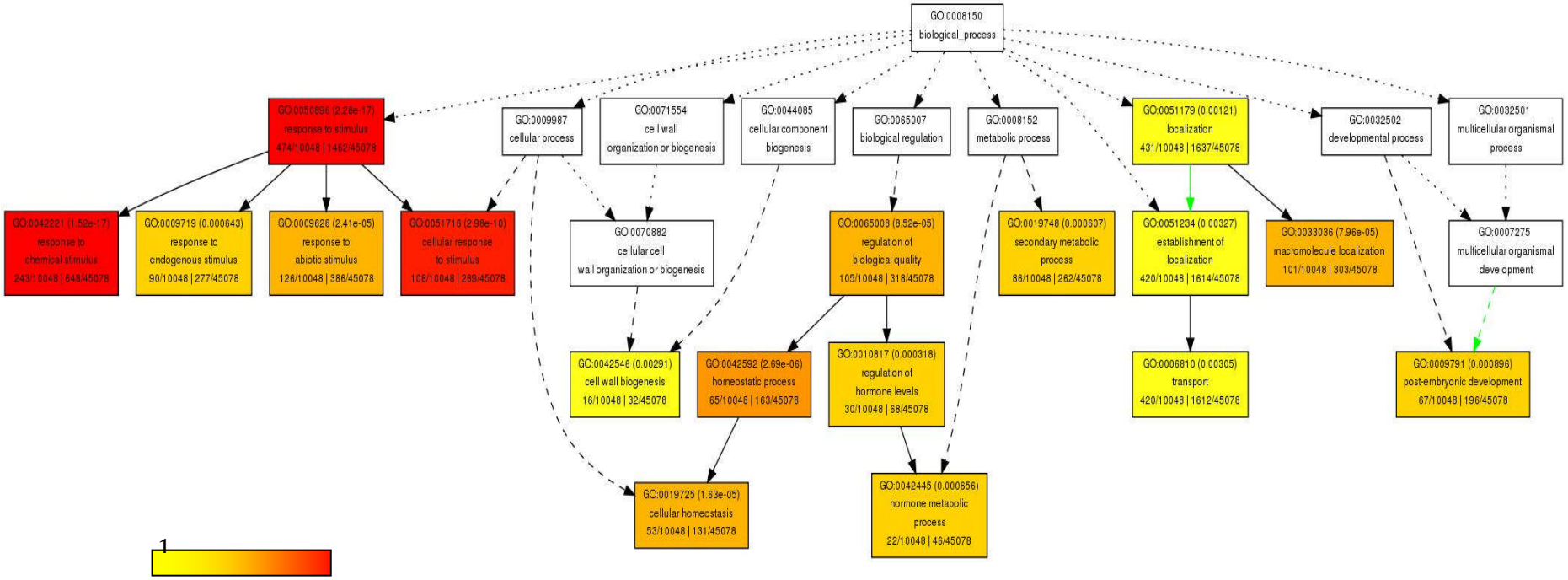


Figure 3-12 Gene ontology enrichment analysis for genes significantly differentially regulated in roots under drought stress. The number of genes associated with each biological process in the DEGs list was compared to the proportion of genes on the microarray in the same biological process using singular enrichment analysis by agriGO. Each square represents a GO category followed by significance in the brackets, name of each GO category, ratio of genes in the list and ratio of genes on the microarray belonging to that category. The squares are colour coded for level of significance; yellow is significance level 1 and red is most significant, level 9. The green arrows show negative regulation.

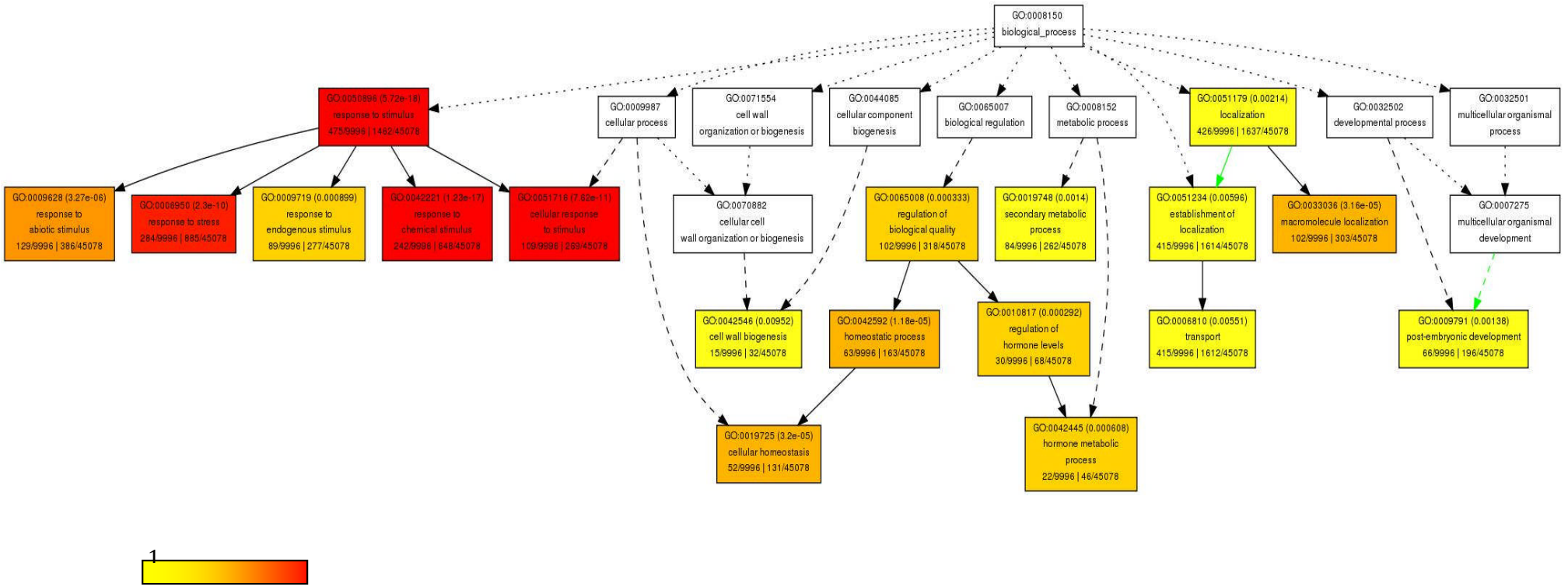


Figure 3-13. Gene ontology enrichment analysis for genes significantly differentially regulated in roots under simultaneous multiple stress. The number of genes associated with each biological process in the DEGs list was compared to the proportion of genes on the microarray in the same biological process using singular enrichment analysis by AgriGO. Each square represents a GO category followed by significance in the brackets, name of each GO category, ratio of genes in the list and ratio of genes on the microarray belonging to that category. The squares are colour coded for level of significance; yellow is significance level 1 and red is most significant, level 9. The green arrows show negative regulation.

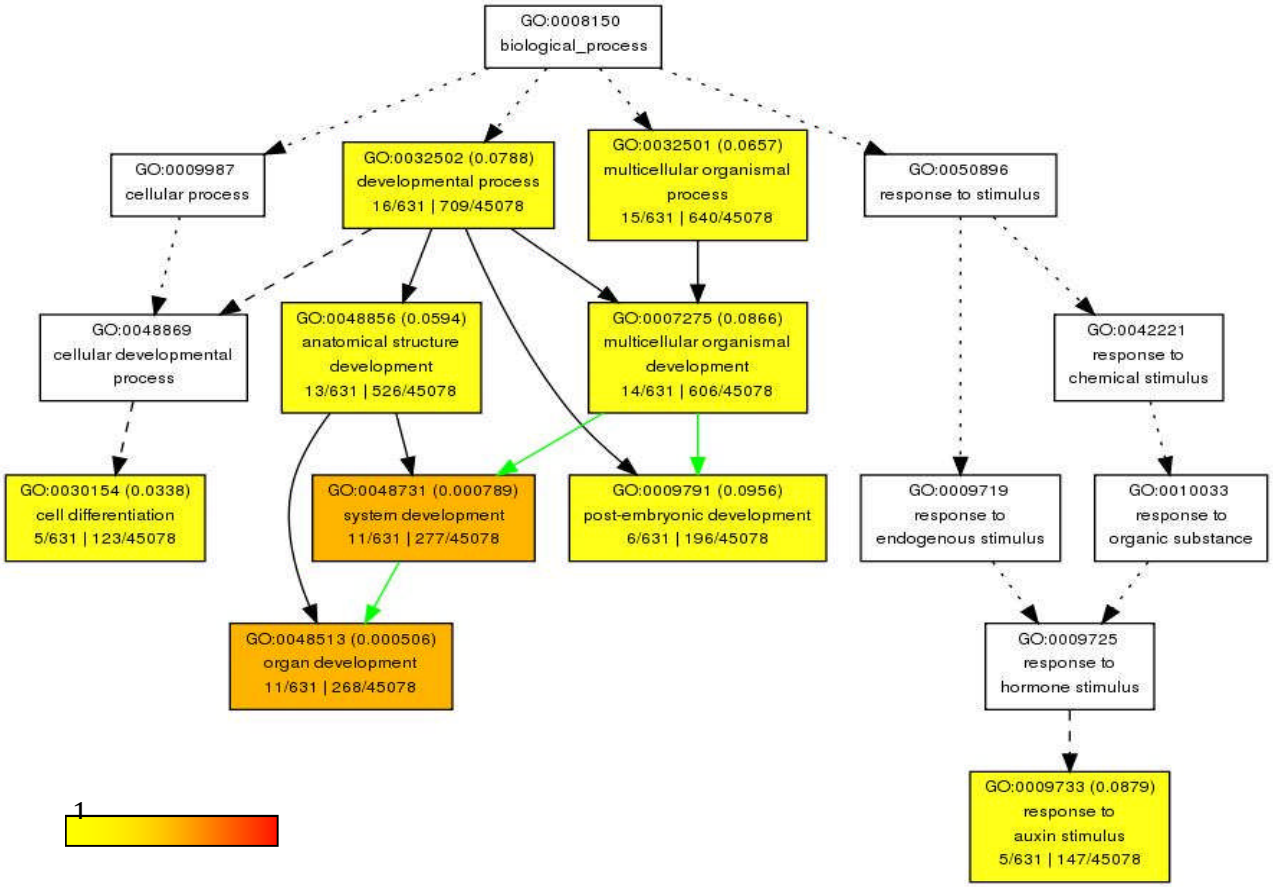


Figure 3-14. Gene ontology enrichment analysis for genes significantly differentially regulated in roots under nematode stress. The number of genes associated with each biological process in the DEGs list was compared to proportion of genes on the microarray in the same biological process using singular enrichment analysis by agriGO. Each square represents a GO category followed by significance in the brackets, name of each GO category, ratio of genes in the list and ratio of genes on the microarray belonging to that category. The squares are colour coded for level of significance; yellow is significance level 1 and red is most significant, level 9. The green arrows show negative regulation.

3.4. Discussion

3.4.1. Microarray experiment and data analysis

A comprehensive investigation of systemic and local transcriptomic responses of rice towards drought and nematode stress, in isolation as well as in combination, was conducted using Affymetrix Rice GeneChip® arrays. The Affymetrix Rice GeneChip® array provides maximum coverage of the rice genome, representing 57,381 transcripts from both japonica and indica type cultivars. Databases with information about probe sequences, latest gene annotations for each probe and gene ontology for most of the identified, protein-expressing genes are available for the Rice GeneChip®. The whole experiment was repeated three times over a period of six months, to ensure that the stress treatments were controlled and replicable and the results arising will be specific to gradual drying of plants and a standard nematode infection. Each treatment included ten plants per replicate to cover variability arising due to differences among the plants. Appropriate biological replication for microarray is a pre-requisite for publication of the study is a vital part of minimum information about a microarray experiment (MIAME) (Brazma *et al.* 2001).

The probe intensities of hybridised arrays from all three replicates of each treatment were compared to all other arrays to determine the correlation between them. The replicates for all three treatments as well as the control plants show poorer correlation in the leaf tissue than the root tissue (Figure 3-4A). The plant leaves sense and respond quickly to the environmental changes around them. Thus, a slight variation in the environment at the time of sampling could have resulted in the particular contrast in the third replicate. The replicate arrays for root samples for all treatments show a higher correlation to other replicates of the same treatment (Figure 3-4B). The array replicates for drought and simultaneous multiple stress cluster in one group whereas the control and nematode stress arrays form the other group. Entire root tip lengths rather than dissected gall tissue were collected to maintain the focus of the study on exploring the systemic effect of the nematode infection, rather than the specific expression changes occurring in the developing feeding site. Use of whole root tips would have diluted the nematode-specific responses and thus clustered these samples with those from control plants. Drought has a major effect on the root physiology and thus the multiple stress array replicates show a higher correlation with the drought stress array replicates.

The systemic gene expression changes induced by pathogen attack can affect genes with a very low level of expression (Puthoff *et al.* 2003), therefore to detect these expression changes a probe summarisation algorithm that accurately selects genes at the lower end of the expression range was preferred. PLIER16 is sensitive and accurate towards lower expression values, it can also work accurately over a range of expression values without losing precision, maintaining a fine balance between the two aspects (Irizarry *et al.* 2003). It runs an optimisation procedure that makes sure the difference in PM and MM is non-negative; this also leads to near zero values for absent probes (Therneau and Ballman 2008). To generate a probe summarisation these values are spiked with a constant (16) followed by logarithmic transformation. This maximises the sensitivity for low expression (Affymetrix 2005).

Statistically strong experimental designs with appropriate replicates and the use of robust commercial microarrays, has increased the accuracy and reliability of expression data from a microarray experiment and largely bypassed the need to conduct corroborative studies. The high correlation between the expression changes identified by the microarray and the qPCR in this study has further supported the evidence of increased reliability and accuracy of the commercial arrays.

3.4.2. Identification of differentially expressed genes

The criteria for identifying DEGs in any study depends on the nature of the study and the biological aim of the experiment. Often, studies use the fold-change difference in expression of genes between control and treatment samples as the simplest and intuitive measure to identify DEGs. An arbitrary threshold of the expression values is applied on log2 expression values, e.g. ± 2-fold and the genes qualifying for that threshold are treated as DEGs (Draghici 2010). However, high fold change in any of the genes does not necessarily mean that the gene is significantly influenced by the treatment imposed. Due to the presence of various underlying variations in microarray procedure, it is not sufficient to rely on the mean fold change alone (Leung and Cavalieri 2003). It is essential to use statistics to calculate the probability of an observed fold change being significant and to control the false positives. Replication is again helpful to impart a greater strength to the statistical tests performed to identify DEGs (Lee *et al.* 2000). When more than two conditions are compared in an experiment, it is favourable to perform an analysis of variance (ANOVA). Testing more than one hypothesis in an

experiment leads to an increased false positive rate and thus applying a multiple testing correction is essential to control false positives (Leung and Cavalieri 2003; Benjamini and Hochberg 1995). Use of a sensitive and accurate probe summarisation method accompanied by the multiple testing correction drastically reduces the false discovery rate and the scope of type I error (Affymetrix 2005; Benjamini and Hochberg 1995). The treatments in this study were compared with controls and among themselves by a one-way ANOVA, followed by the Benjamini-Hochberg FDR multiple testing correction. Genes qualifying above the threshold of a two-fold expression change with a $p \leq 0.05$ in any of the treatments when compared to controls were designated as DEGs. The lists of DEGs were compared to obtain genes expression changes that were common between all three stress treatments and the ones which were specific to any of the stresses individually or on simultaneous application.

3.4.3. Transcriptomic response to drought stress

The drought stress experienced by the plants in a field is gradual and is different from the dehydration stress commonly induced in the laboratory by air-drying, mannitol or PEG methods. Similarly, plant responses to a gradual, progressive drought will be different from those to the acute, sudden dehydration induced in the laboratory. This study succeeded in reproducing a vegetative drought stress that was replicable and consistent between replicates. Thus, this is the first study to provide comprehensive genome-wide transcriptomic profiles of rice grown in soil, under field-like conditions, for both leaf and root tissue at the tillering stage (vegetative drought stress). Leaves show an immediate physiological response to drought that leads to the speculation of leaf tissue undergoing massive drought-responsive transcriptomic changes (Wopereis *et al.* 1996). On the contrary, in this study the root tissue showed a much stronger transcriptomic response to drought (4647 DEGs) than the leaves (490 DEGs) (Figure 3-6A-B). Nearly 10 % of the total transcripts on the chip were drought-responsive in roots, whereas leaves showed differential expression of only 1 % of the transcripts on the array. Due to practical difficulties associated with sampling root tissue, our knowledge on the root responses towards drought occurring in field-type conditions is limited. The only other study that has investigated the rice root transcriptome under drought stress in realistic drought conditions identified only 1154 genes differentially expressed in roots at tillering stage. A smaller number of genes was identified as

drought responsive in that study due to the very high expression cut-off that was employed, i.e. five-fold expression change (Wang *et al.* 2011). In spite of the high cut-off, in that study, leaves at tillering stage showed differential regulation of 878 genes. We have identified 476 drought responsive genes in leaves at tillering stage. The lower number seen in the current work may be a result of the poor correlation between the probe intensities of the hybridised replicate arrays in leaves (Figure 3-4). This along with the probe-specific summarisation method and multiple testing corrections would have discounted the genes that showed very high variability between the replicates. Similar responses have been reported when comparing drought-tolerant and drought-susceptible cultivars under long-term drought stress, in a soil-based model. The drought susceptible group in that study included the cultivars Nipponbare and Taipei and the results are cumulative of responses in both the cultivars. Drought stress induced 219 genes and repressed 474 genes, exclusively in the susceptible cultivars (Degenkolbe *et al.* 2009).

In the whole plant 2,274 genes were induced and 2,612 genes were repressed in response to the drought stress. Of the induced genes, 9.23 % were common between roots and leaves and only 1.03 % of the repressed genes were common between the two tissues at tillering stage. The results are similar to the results obtained by spatial and temporal studies of the near isogenic lines, emphasising the fact that there is a tissue specific response to the drought stress and a very small general response as a whole (Wang *et al.* 2011).

Drought responsiveness in leaves

Nine genes were differentially expressed in leaves, exclusively in response to drought (Table 11). Eight of these genes were only expressed in leaves whereas the putative aspartyl protease was also up-regulated in roots in response to drought and multiple stresses. This belongs to a family of 96 aspartyl proteases in rice, involved in protein degradation (Chen *et al.* 2009). Members of the aspartyl protease family in Arabidopsis are known to be involved in ascorbic acid-mediated drought tolerance and salt tolerance (Gao *et al.* 2011)

One of the two “expressed proteins” regulated specifically in drought-stressed leaves is the abiotic stress responsive LOC_Os03g50870 that is similar to the NAC domain containing protein 1 in Arabidopsis and NAC1 transcription factors in rice and other

monocotyledons. A root-specific OsNAC10 induces drought tolerance and grain yield in rice (Jeong *et al.* 2010). The second “expressed protein”, LOC_Os10g42250, is also known to be stress responsive and shares 72 % sequence similarity with AMMECR1 in Arabidopsis that is characterised as abiotic stress responsive. The glutamate dehydrogenase, 4-α-glucanotransferase and hexokinase are all involved in carbohydrate metabolism. The long cell-linked locus protein and the vacuolar sorting receptor proteins are involved in protein modification. Thus, drought majorly affects carbohydrate metabolism as well as protein modification and degradation processes.

The GO enrichment analysis of all the drought responsive genes in leaves is characterised by the carbohydrate metabolic process, amino acid biosynthesis and osmotic stress responses. Drought stress also influences macromolecule translocation, specifically of lipids and also majorly influences cellular nitrogen metabolism.

Drought responsiveness in roots

The drought stress has a drastic influence on the roots of rice plants, the expression of a higher number of genes (4645) corresponding to different biological processes is altered, mainly repressed (Table 15). 464 genes were specific to drought stress only, 20 % of these were up-regulated and the remaining 80 % were down-regulated. The most significantly induced genes included drought responsive genes identified by laboratory models of acute drought stress i.e. dehydration, ABA application and air-drying (Rabbani *et al.* 2003; Wang *et al.* 2011; Moumeni *et al.* 2011) (Table 14). DNAk family proteins (Lee *et al.* 2011b) belong to the HSP70 family and are the chaperones involved in protein folding in all cellular compartments (Sarkar, Kundnani and Grover 2013). Induction of HSP70 proteins in transgenic plants imparts improved tolerance to heat, water and salt stress (Wang *et al.* 2004). The HSP70 plays a broader role in stress responses by modulating signal transductors such as protein kinases A and protein kinases C along with protein phosphatases (Ding, Tsokos and Kiang 1998). The cellular overview of the genes differentially expressed under drought stress shows that it changes expression of genes involved in all cellular processes. Generally, plant thionins are identified as pathogenesis related proteins known for their antimicrobial activity but, recently, an ABA-responsive thionin has been identified in Ginseng (Jwa *et al.* 2006; Lee *et al.* 2011b). Thus, it is likely that the THION14 is the first rice thionin identified in response to drought stress in roots. Functional analysis shows that genes involved in

biotic stress response, cell cycle, reduction of heme group and development were repressed whereas the genes involved in response to various abiotic stresses and reduction of oxidative species were up-regulated. Repression of biotic stress responsive genes strengthens the hypothesis that the abiotic stress responses suppress biotic stress responsive genes and make plants susceptible to biotic attack (Atkinson, Lilley and Urwin 2013) . Induction of abiotic stress responsive genes identified by the dehydration model and ABA application in rice confirms that the gradual drought stress imposed on plants in this study was sufficient to generate responses similar to the acute stress methods. At the same time, strong induction of novel drought responsive genes suggests that the plant responses towards prolonged gradual drought stress experienced in fields are much more complex than that induced by laboratory models of drought stress.

3.4.4. Transcriptomic response to nematode stress

A compatible plant parasitic nematode infection requires reprogramming of the infected host cells to enable establishment of a successful feeding site; this also triggers systemic stress responses in the other parts of the plant. Many studies have focused on understanding gall-specific transcriptomic changes induced because of nematode feeding by dissecting the galls or micro-aspirating the contents of the gall tissues, but to understand the effect of the nematode parasitism in combination with any other stress, it is essential to obtain more knowledge of the response of the plant as a whole towards the established infection. Root tips of infected and control plants were sampled along with the leaves to explore the transcriptomic changes in root cells along with the systemic changes in leaves. A very small number of genes in the nematode-stressed plants satisfied the cut-off criteria of the significant two-fold expression change ($p \leq 0.05$). Altogether in the leaves and roots, only 19 genes were identified as DEGs with the set criteria (Figure 3-5A-B). Only three probe sets showed significant nematode-specific regulation in roots. Only one of these three has a locus assigned to it (LOC_Os09g26890) and represents an expressed protein that shows 60 % sequence similarity with the mitochondrial transcription factor family protein in Arabidopsis. The number of differentially regulated genes obtained is similar to the results obtained in the study performed by (Puthoff *et al.* 2003). In that case, whole Arabidopsis root systems infected with cyst nematodes were compared to the root systems of non-infected plants. The expression of any of the nematode-responsive genes that were expected to be

induced locally within the gall tissue or feeding cells would have been diluted by the non-gall root tissue in the root tip. The heat-maps produced with probe intensities of hybridised arrays, for leaves and roots show that the samples obtained from the nematode infected plants correlated to control plant samples. This will explain the small number of significantly regulated genes identified from the treatment and the low magnitude of their change in expression.

The nematode infection influenced gene expression more strongly in roots (840 genes at $p \leq 0.05$ no FC) than in leaves (88 genes at $p \leq 0.05$, no FC) (Figure 3-6A-B). The systemic responses were tissue specific and only two unknown proteins, one with a putative epigenetic and regulatory function, were common between the roots and the leaves. Almost 50 % of these DEGs in roots were induced and the other half was repressed (437 up-regulated and 403 down-regulated) but in leaves 80 % of DEGs were induced and only 20 % repressed (70 up-regulated and 18 down-regulated).

Nematode responsive genes in leaves

Plant parasitic nematodes are known to induce expression of pathogenesis-related proteins along with salicylic acid-mediated systemic acquired resistance in the non-infected tissue of the plant. The information on systemic changes in nematode infection is very limited. Studies have focused on individual hormone response pathways to explore the systemic responses during RKN infection. This study is the first study highlighting the transcriptomic profile of systemic changes in the leaves of rice plants infected with the RKN *M. graminicola*. Systemic responses in rice leaves at tillering stage, at an early infection point (4 dpi) are subtle and are mainly characterised by the induction of genes involved in general stress responses (Table 10). These genes include members of the LTPL family (LTPL 12, LTPL 56, LTPL 22 and LTPL23), cytochrome P450, protein phosphatase 2C and OsRCL2-7. The genes that satisfied both the expression level and significance cut-off were common between all three stresses, so are likely to be involved in general stress responses or are wounding induced. The genes with significant expression change and fold change above 1.5 include uncharacterised proteins. The changes in expression were not as significant as for the other two stresses. When ranked in order of significance, the most significant expression change was observed in cold acclimation protein WCOR413 (Table 12). Apart from its role in cold acclimation and abiotic stress responses, it has also been identified in expression

analysis studying response to jasmonic acid (Zhang *et al.* 2010; Gao *et al.* 2008; Jung *et al.* 2007). Another significant expression change was observed in expression of a hydrolase gene, member of nudix family of cytosolic protein. In Arabidopsis, a nudix hydrolase *NUDT7* regulates plant immunity in SA independent manner. The *nudt7* mutants suffer from SA induced cell death and growth retardation. (Bartsch *et al.* 2006). In a compatible infection the nematode suppresses the defence responses and modulates the plant response to their own benefit without causing much damage to the plant as a whole (Caillaud *et al.* 2008). As RKN infection is a major problem in aerobic and upland rice cultivation, where nurseries are generated and seedlings are transplanted into the flooded fields, there is less chance of having a significant influence on plant performance due to an early RKN infection (Kreye *et al.* 2009). The effect may manifest in later stages of plant growth and nematode development, and might lead to compromised yield (discussed in chapter 5).

Nematode responsive genes in roots

Previous studies have focused on the transcriptomic changes in gall tissue and/or feeding cells during a RKN infection; little is known about the effect of RKN infection on the root as a whole. We studied the changes in root tips pertaining to a successful RKN infection. Roots were characterised by a higher magnitude of transcriptomic changes than leaves, mediated by an equal share of induced and repressed genes. The genes with the most significant expression change are the members of signalling cascades and stress responsive families (Table 18). Very small (1.17 fold change) but highly significant change was observed in expression of IQ calmodulin binding domain-containing protein in the roots of the nematode stressed plants. Proteins with a calmodulin binding domain are targets for calmodulin signalling and the members of this family are involved in vital processes like metabolism, phosphorylation, ion transport, cytoskeleton function and DNA binding (Snedden and Fromm 1998). Calmodulin, a calcium modulated protein, has no catalytic property of its own, but it modulates the function of other proteins by physically interacting with them. A calmodulin binding protein, calmodulin-regulated transcription factor, acts as a negative regulator of SA-mediated defence responses and also mediates disease resistance by interacting with a key signalling component of R gene-mediated plant immunity against bacterial and fungal pathogens in Arabidopsis and rice (Poovaiah *et al.* 2013). Dirigent

proteins involved with lignin biosynthesis show significant > 2-fold expression changes (Schlink 2011). Although their function is not very clear they show wounding-induced expression in leaves attacked by herbivores (Schlink 2011). Strong down-regulation of dirigent proteins is also seen in young galls generated by *M. graminicola* in rice (Kyndt *et al.* 2012a). The rice glutathione S-transferase shows biotic stress responsive expression in response to the fungus *Magnoporthe grisea* and *Striga herminthica* parasitism (Jain, Ghanashyam and Bhattacharjee 2010). Significantly affected classes of genes were responsible for cell differentiation, organ development and also response to auxin stimulus. The transcriptome of root tips is generally characterised by these biological processes but significant over-representation of the genes related to these processes in only the nematode infected plants supports the hypotheses that the formation of giant cells and galls is a result of programmed re-differentiation (Caillaud *et al.* 2008). It also strengthens the vital role of auxin signalling in successful RKN infection (Grunewald *et al.* 2009). These functional categories are also over represented in galls in a recent study exploring the transcriptomic responses of rice towards RKN *M. graminicola* infection using mRNA seq. Gibberellic acid biosynthesis was unaffected in whole root tissue, indicating that the up-regulation observed in the mRNA sequencing study is gall specific (result not shown here). Similar to the gall cells, the whole root system also shows down-regulation of a dirigent protein. Most of the other RKN responsive transcripts identified in our study are expressed proteins of unknown function, thus promising a vast opportunity of identifying novel responses and interactions that might be essential for RKN infection.

3.4.5. Transcriptomic changes in response to simultaneous drought and nematode stress

Plant responses to two simultaneous stresses can be interactive, additive or antagonistic of the responses seen towards any of those two stresses individually (Atkinson and Urwin 2012). The damage caused by a combination of two abiotic stresses, especially heat and drought, is more alarming and the plant responses under these conditions have been studied in detail in model plants as well as in crop species (Hewezi, Léger and Gentzbittel 2008; Rizhsky, Liang and Mittler 2002; Dobra *et al.* 2010; Jiang and Huang 2001; Mittler 2006). Investigating various abiotic stresses in combination, (Rasmussen *et al.* 2013) stated that the effect of a combination of two abiotic stresses on a plant can

be independent, cancelled or combinatorial. The effect of various biotic stresses in combination have led to an understanding that apart from general responses, plants tackle each stress in a different way (Li *et al.* 2006a). Responses to various biotic stresses have been compared to those for abiotic stresses with the goal of determining the general stress responses of plants but such comparisons fail to determine how the plant will respond to simultaneous application of a biotic and an abiotic stress (Narsai *et al.* 2013). Our knowledge of the plant responses to a combination of an abiotic and a biotic stress is very limited. Transcriptomic investigations of the combined effect of a biotic stress, *Aspergillus parasiticus*, and an abiotic stress, drought, in peanut, showed that the response to the combinatorial stress was more similar to the drought response alone with a very small proportion of multiple stress-specific responses (Luo *et al.* 2005). Similar results were seen in Arabidopsis plants simultaneously exposed to dehydration and infection with the cyst nematode *Heterodera schachtii* (Atkinson, Lilley and Urwin 2013). The plants under simultaneous biotic and abiotic stress showed a tailored response to a combination of multiple stresses. The plant prioritises a more challenging abiotic stress over a less harmful biotic attack. We saw similar responses, the transcriptomic changes to simultaneous stress were dominated by drought responsive changes with a small proportion of unique simultaneous multiple stress specific responses.

Simultaneous multiple stress responsive genes in leaves

95 % of the responses in multiple stressed leaves were similar to those seen in response to the drought stress and only 5 % were unique multiple stress-specific responses (Figure 3-4A-3-4B). The systemic responses to nematode infection were subtle and they did not overlap with the multiple stress response apart from the general stress responses that were induced in all three stresses. The simultaneous stress responsive changes seen in this study have led to identification of novel members of gene families that are known for cross-talk in biotic and abiotic stress responses (Table 13). One of the genes highly induced specifically under multiple stress is LTPL 11, that is a previously uncharacterised member of the family of proteins that are stress responsive and involved in pathogenesis as well as abiotic stress response in rice (Vignols *et al.* 1997). Various members of the LTPL family have shown defence related roles in other plants. In Arabidopsis, they impart salicylic acid-mediated SAR in response to *Pseudomonas* and

Peronospora parasitica infection (Maldonado *et al.* 2002). Transgenic expression and exogenuis applicaiotn of purified LTPL II in tobacco and Arabidopsis reduces the lesion size under *Pseudomonas syringae* infection (Molina and García-Olmedo 1997). They might play an important role in signal transduction by being a co-signal or a translocator of secondary messengers like jasmonic acid (oxylipins), phosphatidic acid and other lipid molecules induced at time of pathogen attack (Maldonado *et al.* 2002). Thus, the LTPL11 identified is a potential candidate for mediating cross-talk between SAR and the ABA response towards drought. Another large family of genes involved in cross-talk of biotic and abiotic stress is the cytochrome P450 family with 328 genes and 99 pseudogenes in rice (Nelson *et al.* 2004). Two different members of this family were up-regulated specifically in response to the multiple stresses. The cytochrome P450s in Arabidopsis mediates cross-talk between the abiotic and the biotic stress responsive hormone pathways. They are involved in catabolism of ABA, the major abiotic stress responsive hormone, deactivation of gibberellic acid and negative regulation of jasmonate pathway (Koo, Cooke and Howe 2011). One of the cytochrome P450s identified shares 40 % sequence similarity with the Arabidopsis cytochrome P450 CYP741A1 that deactivates gibberellins and mediates plant growth. Over-expression of the Arabidopsis cytochrome P450 CYP741A1 leads to stunted growth and the highly induced levels of the same in our study can justify the stunted growth of plants subjected to simultaneous stress (Zhang *et al.* 2011). Up-regulation of the alpha-amylase responsible for degradation of sucrose and the down-regulation of starch synthase in multiple stressed plants indicate that multiple stress significantly modulates carbohydrate metabolism. Drought stress affects alpha-amylase in leaves and thus modulates sugar metabolism (Jacobsen, Hanson and Chandler 1986). Sucrose is required for plant growth but it also acts as a signalling molecule by modulating a proton-sucrose symporter (Gupta and Kaur 2005). The alpha-amylases are secreted from cells because of which they are involved in hormonal cross-talk and signalling. The Arabidopsis orthologue of up-regulated gene xyloglucan galactosyltransferase KATAMARI1 infers salt stress tolerance and the rice gene might have a unique function in abiotic stress along with the biotic stress (Li *et al.* 2013b). The remaining identified genes like the solute carrier family 35 member F1, oxidoreductase and 4,5-DOPA dioxygenase extradiol also show only simultaneous stress-responsive changes in

expression and thus can be candidates for modelling plant responses to simultaneous abiotic and biotic stresses.

Simultaneous stress responsive genes in roots

With both of the simultaneous stresses acting on roots locally, many more genes were differentially expressed in roots in response to the simultaneous drought stress and RKN infection. Similar to the drought stress, nearly 10 % (4,480) of the genes on the chip had a two-fold expression change at a significant level ($p \leq 0.05$) in the roots. The transcriptomic changes were tissue specific with only 5 % overlap between the roots and the leaves. A total of 297 genes showed multiple stress specific regulation. Contrary to the pattern seen in drought stress responses, in response to the simultaneous stress 75 % of the genes were induced and 25 % were repressed (Figure 3-4A-3-4B). Two members of the cytochrome P450 family showed two-fold induction in leaves in response to simultaneous multiple stresses, but in roots two other members of the same the family showed a highly significant ($p \leq 0.001$) 13-fold repression. Increased levels of various cytochrome P450s are associated with increased disease tolerance in plant species (Morant *et al.* 2003). Differential regulation of cytochrome P450 s in biotic, abiotic and oxidative stress suggests that they play an important role in interplay between different signalling and hormonal pathways (Narusaka *et al.* 2004). A detailed profiling of rice cytochrome P450s in response to various stresses would be beneficial to understand the specific role of each member of this family in various stresses. Another significantly repressed gene, wall-associated kinase, plays an important role in resistance to rice blast disease. Plants with constitutive expression of *WAK1* develop resistance to a compatible blast fungus race (Li *et al.* 2009a). They are membrane embedded receptor-like proteins, that have undergone recent evolutionary events in rice to evolve as disease-responsive receptor kinases (Shiu *et al.* 2004). Their role in multiple stress or abiotic stress has not been previously reported, making response to simultaneous biotic and abiotic stress a novel function of known wall-associated kinase 1. Interestingly, various nodulins have been identified throughout our analysis. One of the nodulins, MtN3 (LOC_Os01g42090), has been found antagonistically regulated in the roots and in the leaves of drought and multiple stressed plants. Another nodulin (LOC_Os09g25810) is repressed in roots under drought conditions whereas nodulin MTN3 (LOC_Os03g22200) is induced in roots.

Study of simultaneous abiotic and biotic stress in rice indicates that under a simultaneous attack of two major stresses, plant responses are governed by the abiotic stress. The rice plants respond more severely to the drought stress than to RKN infection. It might be because under severe vegetative drought stress, the plant is struggling for survival and reproduction and thus a vast array of gene expression changes are seen in order to adopt strategies for drought avoidance. Whereas, the moderate RKN infection at the vegetative phase of the plant is not a fatal stress for plants and is treated as a secondary stress when in combination with a more challenging stress like drought. The simultaneous stress response in rice is characterised by an unique set of genes that are not differentially regulated when any of the two stresses act individually on the plant, emphasising that the response to a combination of stresses is not additive but is interactive of the responses seen under the influence of any of the stresses singularly. The expression analysis using microarray analysis in rice to identify stress responsive changes in response to drought, nematode and simultaneous drought and nematode stress has led to identification of new members of known stress responsive families. It has also highlighted new gene families and uncharacterised expressed proteins that might have a significant role in the stress responsiveness of rice.

4 Chapter

Functional analysis of candidate genes in rice

4.1. Introduction

The rice genome has 27-65.7% gene duplication, resulting in a large number of genes with redundant function. In spite of having a fully sequenced genome and a range of genetic techniques available with which to elucidate function, there are fewer genes with functional annotations in rice than in the model dicot plant *Arabidopsis thaliana* (Jung, An and Ronald 2008). Thus, the challenging task after a transcriptomic study is to determine the biological relevance of the differential gene expression seen towards the set of conditions studied. Extensive genetic and molecular exploration of the function and nature of the genes found by transcriptomic profiling is required to understand the response of the plant as a whole towards a certain stimulus. With a small number of rice genes functionally annotated, the foremost step is to determine the function of individual candidate genes identified by transcriptomic studies. For functional characterisation of genes, several reverse genetic approaches have been developed and utilized, including insertional mutagenesis, homologous recombination and RNAi suppression of the gene of interest (An *et al.* 2005).

4.1.1. Rice reverse genetics

The insertion mutation is, to date, the most efficient and widely accepted method for elucidating the function of a gene. A foreign DNA fragment integrates at a random site into the genome, this disrupts the gene where the insertion occurs, as well as tagging it for isolation and further analysis. The insertion can be achieved by *Agrobacterium*-mediated transformation or by activation of transposons (Gelvin 2003; Speulman *et al.* 1999). For Arabidopsis, various efficient protocols for *Agrobacterium* transformation have been developed, including seed transformation, floral dip transformation and root transformation (Valvekens, Van Montagu and Van Lijsebettens 1988; Clough and Bent 1998; Zhang *et al.* 2006; Feldmann and Marks 1987). Insertion mutants have been used on a wide scale in Arabidopsis to generate databases that have been used for functional analysis of various genes (Brown *et al.* 2005; Sessions *et al.* 2002; Krysan, Young and

Sussman 1999; Alonso *et al.* 2003). Transformation of rice using *Agrobacterium* co-cultivation protocols has been adopted to produce large populations of T-DNA insertion mutant lines (Hiei, Komari and Kubo 1997; Hiei *et al.* 1994; Shrawat and Good 2010; An *et al.* 2003; Sallaud *et al.* 2004). Apart from T-DNA insertion, the endogenous retrotransposon element *Tos17* has also been used to generate insertion mutants in rice (Hirochika 2001). *Tos 17* is a low-copy number retrotransposon with only two copies in the Nipponbare cultivar. The transposon becomes active under tissue culture and is inactivated in regenerated plants, with 5-30 insertions induced in plants cultured for 6-13 months (Miyao *et al.* 2003; Hirochika 2001). The T-DNA insertions are heritable, stable and non-uniformly distributed over the genome, with rare insertions in repetitive sequences and higher chances of integration in regions with high gene density (Sallaud *et al.* 2004). *Tos17* insertion requires a specific GC content, has a higher preference for insertion in the kinase and resistance genes, and is driven by a palindromic sequence at the site of insertion. This preferential insertion causes rearrangements in the kinase and resistance genes that can alter plant stress response. (Miyao *et al.* 2003). The random insertion of T-DNA fragments or the *Tos17* copies in the genome generally disrupts the function of the gene at the site of insertion and generates a loss-of-function mutant. Although observing functional and phenotypic changes arising from disruption of a certain gene is the best way of revealing its function, knock-out mutants are not effective for genes that show functional redundancy, are involved in early stages of plant development or are vital for plant growth (Jeong *et al.* 2002). A modified T-DNA insert with a strong activator element to form activation tag or gene trap can be used to overcome this problem (Tani *et al.* 2004). The gene and promoter trap system comprise a promoter-less reporter gene *β-glucuronidase* (*gus)* or a green fluorescent protein (*gfp)* integrated in the insert. This insert disrupts the gene it is inserted into but expression of the reporter is driven by the promoter of the disrupted gene (Springer 2000). Spatial and temporal expression of the endogenous gene can be deduced to aid its functional analysis. In activation tagging the reporter genes are fused with a minimal promoter, typically containing a TATA box and transcription start site. These promoters are incapable of driving the expression of the reporter gene themselves but are activated by enhancer elements in a neighbouring region of the insertion. To develop an enhancer trap system, enhancer elements from strong promoters like cauliflower mosaic virus (CaMV) 35S and yeast transcriptional activator and upstream activation sequence

(GAL4-UAS) systems have been used (Wu *et al.* 2003; Springer 2000; Jeong *et al.* 2002). The activation tagging reveals gain-of-function phenotype and results in the quantitative increase of endogenous expression leading to identification of novel genes. A total of 13,450 T-DNA activation lines have been developed using the *GUS* reporter and CaMV 35S promoter based enhancer system (Jeong *et al.* 2002). A library of 29,482 T-DNA enhancer trap lines for Nipponbare was generated using a binary vector conferring resistance to hygromycin under the control of subterranean Clover Mosaic Virus pS4, with a rice *actin1* first intron, *gusA* coding sequence and first 46bp of the 35S CaMV promoter (Sallaud *et al.* 2004). Nearly 50,000 *Tos 17* knock-out mutants were generated and flanking sequences of 42,292 insertions analysed using a thermal asymmetric interlaced PCR protocol (Miyao *et al.* 2003). The sequenced FSTs from these libraries and other rice mutant libraries are catalogued and compiled under OrygenesDB. It is a comprehensive database on rice reverse genetics with interactive interface to search for mutants available for a candidate gene (Droc *et al.* 2006). Out of all the lines integrated in OrygenesDB, 13,928 lines (T-DNA enhancer trap and *Tos 17* knock-out) are phenotypically characterised for forward genetics and represented in Oryza Tag Line database (Larmande *et al.* 2008). The enhancer trap mutants usually influence expression of genes, several kilo base pairs downstream from the site of insertion and thus at times it is hard to conclusively attribute the phenotype to over-expression of any one gene. To study the over-expression of specific rice genes, full-length rice cDNA over-expression lines (FOX) under CaMV35S promoter control were generated in *Arabidopsis thaliana* (Nakamura *et al.* 2007). More than 3000 morphological and phenotypical over-expression mutants for rice genes were obtained using this method and used for elucidating functions of various rice genes (Sakurai *et al.* 2011; Kondou *et al.* 2009; Albinsky *et al.* 2010; Higuchi-Takeuchi, Mori and Matsui 2013).

4.1.2. Evaluating mutant stress-responsiveness

The analysis of insertion mutants has successfully identified genes involved in rice stress responses. Knock-out and over-expression lines often show phenotypic and morphological variations under normal growth conditions. However, for identification of drought responsive genes the loss or gain-of-function mutants are subjected to drought stress assays. The role of a basic leucine zipper (bZIP) protein in

ABA-dependent drought tolerance and salinity tolerance was determined by using over-expression lines and knock-out mutants subjected to a pot-based severe drought assay (Xiang *et al.* 2008). In this case the plants were deprived of water for nearly two weeks, then re-watered and scored on the rate of survival after rehydration. The drought-sensitive rice mutant *dsm1,* a knock-out mutant of mitogen-activated kinase kinase (MAPKK), a member of the MAPK cascade, was used to establish the role of the MAPKK in drought responsiveness mediated by regulating the scavenging of reactive oxygen species (Ning *et al.* 2010). In tissue culture and hydroponic cultures, the air-drying is used to induce drought. Plants are air dried until considerable weight-loss is seen and then placed back on the media. An abiotic stress responsive gene, *OsGSK1* (*glycogen synthase kinase 3 like gene 1),* orthologue to *brassinosteroid insensitive 2* (*BIN2*) was identified by screening T-DNA tagged rice lines in hydroponic growth conditions for cold, heat, salt and drought tolerance (Koh *et al.* 2007). Using a severe drought stress assay followed by re-hydration, such as those described above, provides insight into the ability of a plant to rehydrate rather than assess drought tolerance or susceptibility. Evaluation of true drought responsiveness requires physiological and morphological growth parameters to be monitored under moderate drought. Fewer mutant studies take this approach. Growth profiling of a zinc finger transcription factor mutant, *dst,* showed enhanced drought tolerance by increased stomatal closure and reduced stomatal density (Huang *et al.* 2009b). A drought sensitive mutant, *dsm2*, was identified with lower maximum quantum efficiency of photosystem with reduced photosynthesis rate, biomass and grain yield. The mutant line harboured a T-DNA insertion in a gene coding for β-carotene hydroxylase, that is a precursor for ABA (Du *et al.* 2010).

The role of candidate genes in nematode susceptibility of a plant can be quantitatively assessed by monitoring the rate of nematode infection, development and reproduction on the mutant plants (Green *et al.* 2002). The number of juveniles entering plant roots can be used to access nematode susceptibility of a mutant plant (Atkinson, Lilley and Urwin 2013). The effect of over-expression or loss-of-function on the nematode development can be determined by measuring the effect on nematode developmental stages (Urwin *et al.* 1997a). In field or pot based studies, the nematode susceptibility of the plants is determined by evaluating the nematode reproduction (Roderick *et al.* 2012; Lilley *et al.* 2004). In case of RKN, the number of eggs produced per plant can be

obtained by egg extractions (Lilley *et al.* 2004; Pokharel, Duxbury and Abawai 2012). Whereas, for CN the number of cysts released in soil after infection determine the relative growth rate and fecundity of established nematode infection (Urwin *et al.* 2001).

Insertion mutants for the candidate genes that were differentially regulated in the multiple stressed plants were obtained and screened under stressed condition. The mutant response was characterised under drought, RKN infection and simultaneous stress and compared to wildtype plants under respective stress conditions to determine the role of respective genes in plants stress response.

4.2. Material and methods

4.2.1. Selection of candidate genes and rice mutants

Form the results of microarray analysis, the sub-set of genes differentially regulated between the single stress treatments and the simultaneous multiple stress treatment was chosen. The genes significantly differentially expressed between the drought and multiple stress treatment were focused on, for further analysis (provided in accompanying CD). Out of this list, the novel members of gene families known to be stress responsive or the novel genes with unknown functions were selected. These candidate genes identified were screened for mutant availability in OryzageneDB, using the gene locus. Due to unforeseen difficulties with material transfer agreements, only the mutants available through the Oryza Tag Line database of the Génoplante rice insertion line library could be obtained. Thus, the Nipponbare T-DNA and *Tos 17* insertion mutant lines for genes - LOC_Os11g10460, LOC_Os03g48320, LOC_Os08g10250 and LOC_Os09g13630 were obtained from the Génoplante Consortium, France. Table 20 summarises details of the mutants used.

4.2.2. Growth conditions and stress treatment

The stress treatment model designed for the microarray analysis was used to grow the wild type Nipponbare and mutant plants (Section 2.2 and Section 3.2). The stress treatment groups for mutants *per*, *rpp13* and *shr5* had three plants each, the stress treatment groups for mutant *c2h2zf* had two plants each and the stress treatment groups for wild type Nipponbare plants had five plants each. The plants were maintained under the stress regime for 40 days post nematode infection.

4.2.3. Data collection

The stress treatments were initiated on five-week old wild type and mutant Nipponbare plants at tillering stage, thereafter the growth stages were monitored and recorded for all the plants in each treatment. The water use efficiency of wild type and mutant Nipponbare plants under the stress conditions was determined by gravimetric estimation of transpiration taking place each day per pot (Section 2.3.3). The extent of leaf rolling and leaf drying was recorded throughout the stress treatment using the leaf-rolling index

for drought tolerance (Rice knowledge bank, IRRI). All the plants were harvested 40 days post nematode infection. At the time of harvest, tiller number and number of inflorescences on each plant were recorded and all inflorescences per plant were collected. The inflorescences from each plant were allowed to dry and the total weight of seeds produced per plant was obtained.

The roots were cleaned by gently washing off soil. The plant height was recorded by measuring the length from the base to the tip of the longest green leaf in each plant. The roots and shoots of each plant were weighed separately for obtaining fresh weight. The shoot of each plant was dried at 60 ºC overnight and the dry shoot weight was recorded. Egg extractions were performed on the roots of each plant and the numbers of *M. graminicola* eggs and juveniles per plant were determined (Section 2.2.7.)

4.2.4. Statistical analysis

The data obtained for growth, yield and nematode susceptibility assay were normally distributed. The mean value per plant per genotype was determined for each parameter. The mean for each mutant was compared with that of the wildtype plants under the same treatment and the mean of each treatment was compared with the mean of control plants for each genotype using the Student's t-test. The standard error of the mean was also calculated. All the statistical analysis was carried out using SPSS statistics package19 from IBM.

Gene locus	Function	Mutant name	Plant name	Position	Insertion	Expression in stressed plants		
						Drought	Multiple	Nematode
LOC_Os11g10460	**Peroxidase precursor**	*per*	ABZH10	Promoter	T-DNA	Down	Down	Down
LOC_Os03g48320	**Disease resistance RPP 13- like protein 1**	*rpp13*	ARWD10	Exon	Tos17	Up	Up	Up
LOC_Os08g10250	**SHR5 - receptor-like kinase**	*shr5*	AQVC11	5' - UTR	Tos17	Up	Down	Down
LOC_Os09g13630	**ZOS9-03-C2H2 zinc finger protein**	*c2h2zf*	AERF09	Intron	T-DNA	Down	Up	Up

Table 20 The rice insertion mutants for the candidate genes identified using microarray analysis of drought, nematode and simultaneously multiple stressed plants

The mutants were obtained from Génoplante, CIRAD, France and the information about the mutants was obtained from Oryza Tag Line online database, CIRAD, France. The gene locus (RAP-DB ids) and function were obtained from rice genome annotation project.

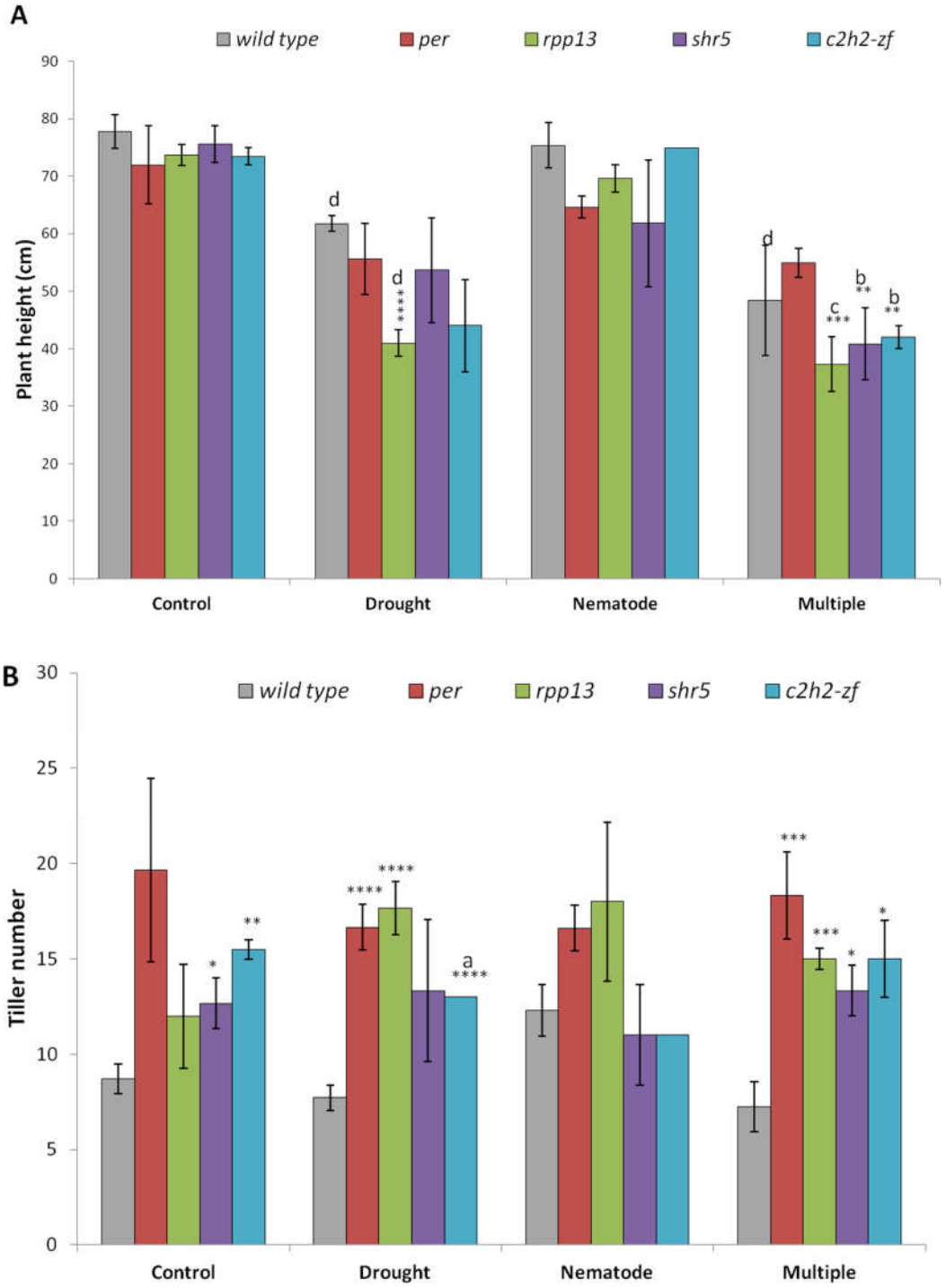


Figure 4-1 Characterisation of the growth parameters of the rice insertion mutants under drought, nematode and multiple stress treatment 40 dpi

The bars represent mean of all plants under the treatment for each mutant type (*per, rpp13, shr5* ; *n* = 3 and *c2h2zf*; *n* = 2) or wild type plants (*n* = 5) . The error bars represent standard error of the mean. The means were compared those of the wild type Nipponbare plants using standard student's t-test (* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.005$ and **** = $p \leq 0.001$). The drought, nematode and multiple stress plants of each mutant type were also compared to the control plants of the same mutant using the student's t-test (a = $p \leq 0.05$, b = $p \leq 0.01$, c = $p \leq 0.005$ and d = $p \leq 0.001$).

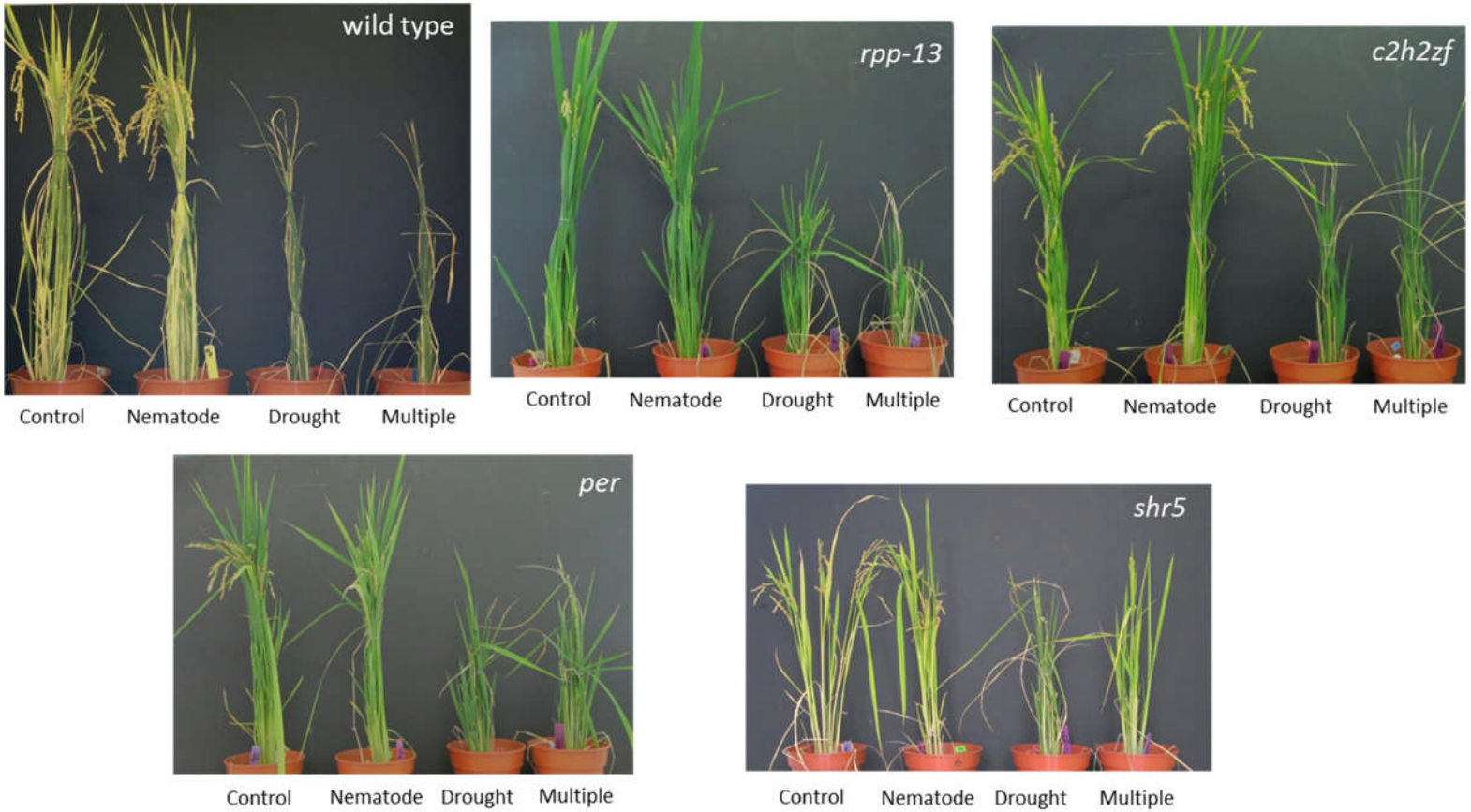


Figure 4-2 Phenotype of the rice insertion mutants *rrp13, c2h2zf, per* and *shr5* and wildtype Nipponbare plants under drought, nematode and simultaneous drought and nematode stress treatment.

The stress treatments were initiated on five-week old plants at tillering. The plants were stressed for 40 days (two subsequent generation of *M. graminicola*) and then harvested. Photographs were taken at harvest.

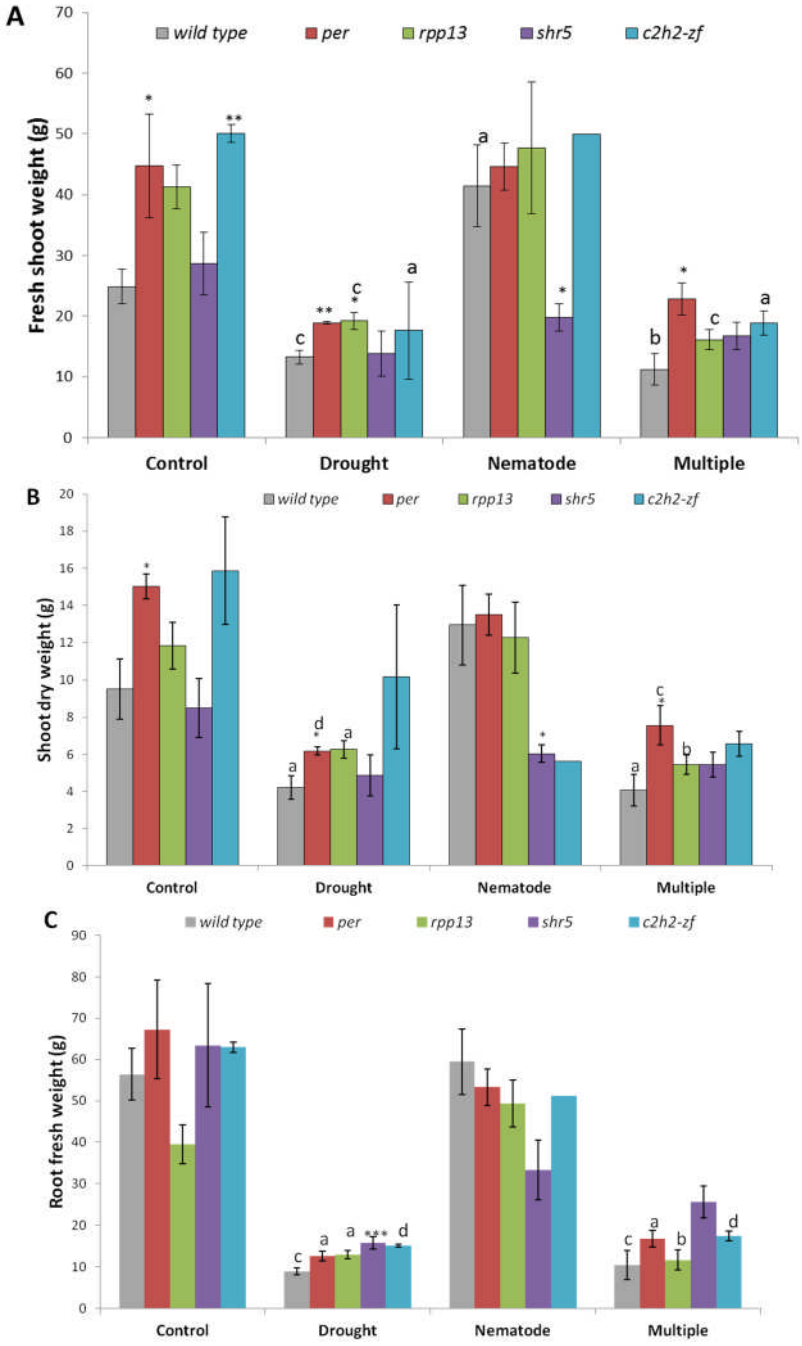


Figure 4-3 Characterisation of growth parameters of the rice insertion mutants under drought, nematode and simultaneous drought and nematode treatment 40 days post infection.

The bars represent mean of all plants under the treatment for each mutant type (*per*, *rpp13*, *shr5* ; n = 3 and *c2h2zf*; n = 2) or wild type plants (n = 5). The error bars represent standard error of the mean. The means were compared to the wild type Nipponbare plants using standard student's t-test (* = $p \leq 0.05$, ** = $p \leq 0.01$ and *** = $p \leq 0.005$). The drought, nematode and multiple stress plants of each mutant type were also compared to the control plants of the same mutant using the student's t-test (a = $p \leq 0.05$, b = $p \leq 0.01$, c = $p \leq 0.005$ and d = $p \leq 0.001$).

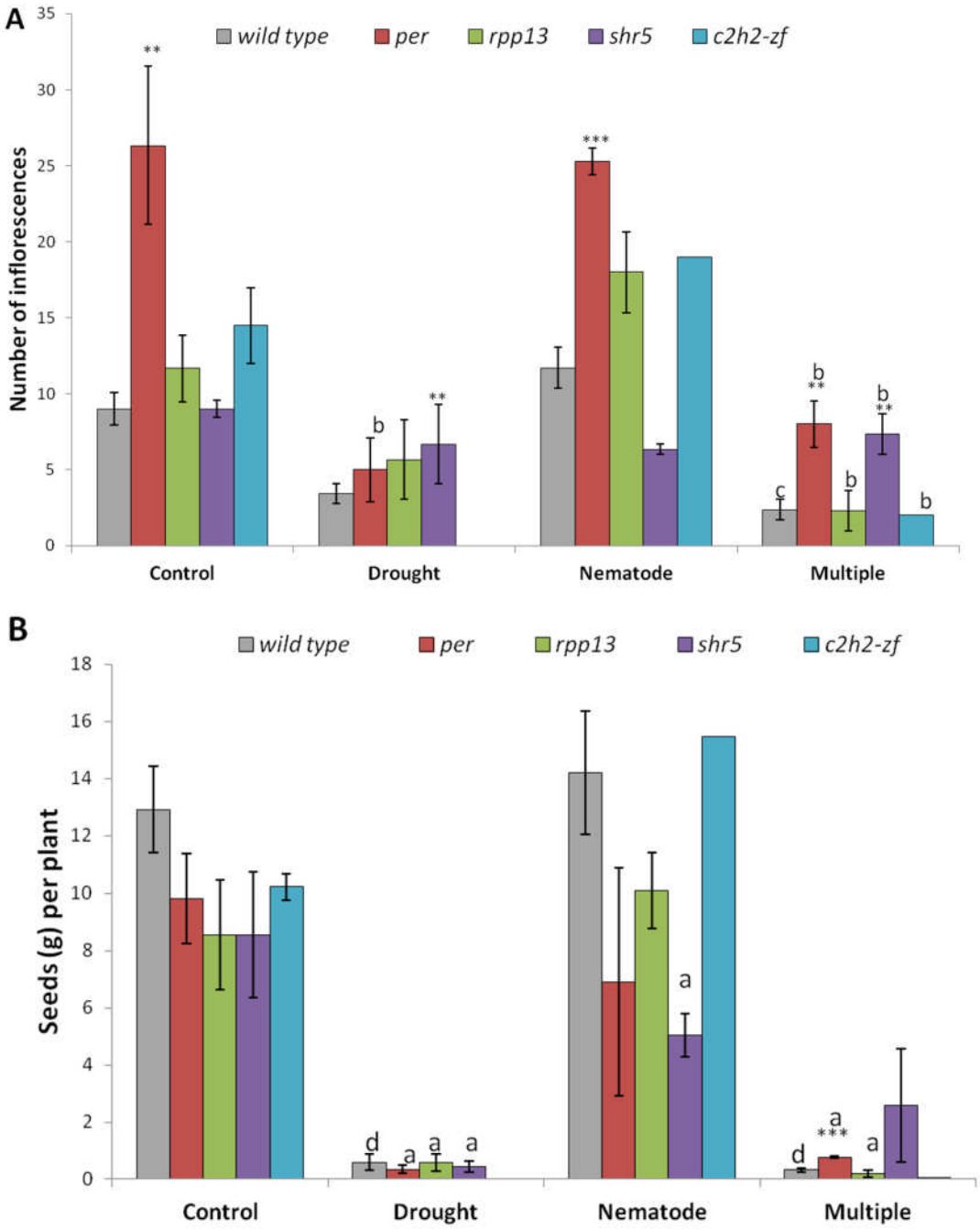


Figure 4-4 Characterisation of yield parameters of the rice insertion mutants under drought, nematode and simultaneous drought and nematode treatment, 40 dpi

The bars represent mean of all plants under the treatment for each mutant type (*per, rpp13, shr5* ; n = 3 and *c2h2zf*; n = 2). The error bars represent standard error of mean. The means were compared to the wild type Nipponbare plants using standard student's t-test (* = $p \leq 0.05$, ** = $p \leq 0.01$ and *** = $p \leq 0.005$). The drought, nematode and multiple stress plants of each mutant type were also compared to the control plants of same mutant using the student's t-test (a = $p \leq 0.05$, b = $p \leq 0.01$, c = $p \leq 0.005$ and d = $p \leq 0.001$).

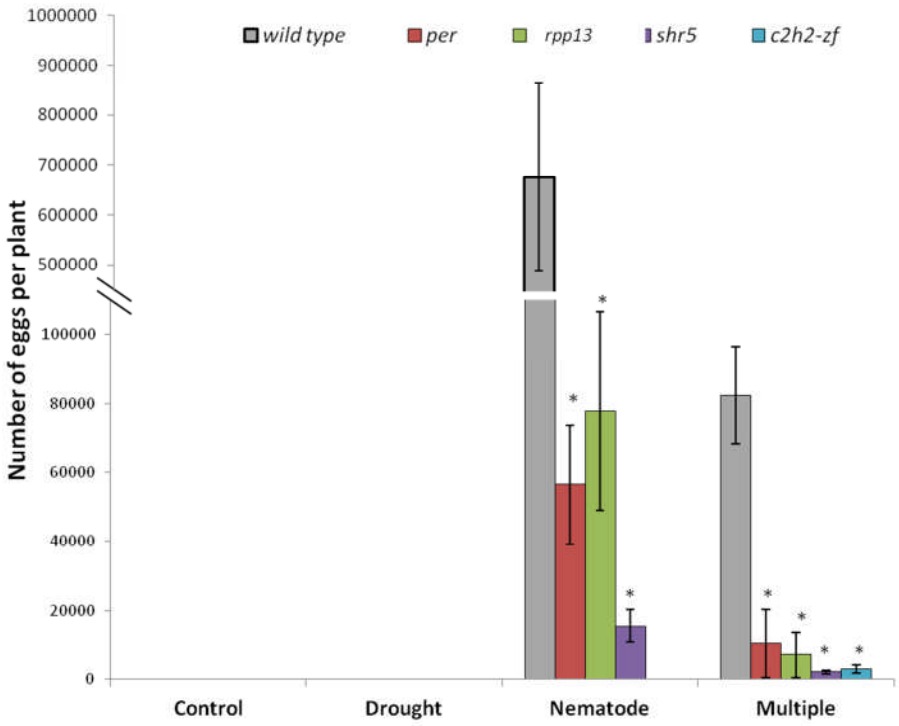


Figure 4-5 Nematode susceptibility of the rice insertion mutants under drought, nematode and simultaneous drought and nematode treatment

The plants under nematode and simultaneous drought and nematode treatment were infected with 3000 second stage *M. graminicola* juveniles and the control and drought stressed plants were mock inoculated with sterile water. The roots were collected on harvest at 40 dpi and egg extraction was performed. The bars represent mean of eggs and second stage juveniles per plant for each mutant type (*per, rpp13, shr5* ; $n = 3$ and *c2h2zf*; $n = 2$) and wild type plants ($n = 5$). The error bars represent standard error of the mean. The means were compared to those for the wild type Nipponbare plants using standard student's t-test
(* = $p \leq 0.05$).

4.3. Results

The insertion mutants for candidate genes were subjected to drought, nematode and simultaneous drought and nematode stress. The effect of the stresses on growth, yield and nematode susceptibility of the mutants under singular stress or a combination of drought and nematode stress was determined.

4.3.1. Growth parameters

Plant height, tiller number, fresh root and shoot weight and dry shoot weight was recorded at harvest to determine the effect of drought, nematode and multiple stress treatment on the mutants and wild type Nipponbare plants. In general, drought and multiple stress treatment had a negative effect on the plant growth parameters. The height of wild type plants was significantly reduced ($p \leq 0.001$) under the drought (61 ± 1.31 cm) and the multiple (48.35 ± 9.57 cm) stress treatments compared to control (77.8 ± 2.93 cm). The *rpp13* mutants had reduced height under drought (41 ± 2.3 cm) and multiple (37.33 ± 4.7 cm) stress when compared to the control plants (73.66 ± 1.85 cm) of the same mutants. The *shr5* (40.83 ± 6.27 cm) and *c2h2-zf* mutants (42 ± 2 cm) under multiple stresses were also reduced in height compared to the control plants of the same mutants. The mutant *rpp13* also showed reduced height under drought ($p \leq 0.001$, 41 ± 2.3 cm) and multiple stresses ($p \leq 0.005$, 37.33 ± 4.7 cm) compared to the wild type plants under drought (61.8 ± 1.31 cm) and multiple stress (48.35 ± 9.57 cm). The mutants *shr5* (40.83 ± 6.25 cm) and *c2h2zf* (42 ± 2 cm) showed reduced height in only multiple stressed plants when compared to the corresponding wild type (48.38 ± 9.57 cm) plants under same treatment. The *per* mutants showed no significant reduction in height under any of the treatments (Figure 4.1.A and 4.2).

None of the three stress treatments had any influence on the tiller number of the wild type Nipponbare plants. Under control conditions, *shr5* ($p \leq 0.05$, 12.66 ± 1.33) and *c2h2zf* ($p \leq 0.01$, 15.5 ± 0.5) mutants had more tillers than the wild type plants (8.71 ± 0.77). Tiller number of all mutants was unaffected by nematode infection alone. Under drought stress treatment, mutants *per* (16.66 ± 1.2), *rpp13* (17.67 ± 1.4) and *c2h2zf* (13 ± 0) produced more tillers than wild type plants ($p \leq 0.001$, 7.71 ± 0.68). Under multiple stresses, all mutants (*per*, 18.33 ± 1.3; *rpp13*, 15 ± 0.57; *shr5*, 13.33 ± 1.3 and *c2h2zf*, 15 ± 2) showed significantly more tillers than the wild type plants (7.24 ± 1.30).

Drought stress significantly decreased tillering in *c2h2zf* (13 ± 0) compared to the control (15 ± 0.5) treatment of same mutant. For other mutants, the different stress regimes did not cause significant change in tillering (Figure 4.1.B).

The fresh shoot weight (FSW) of wild type plants was significantly reduced by drought (13.23 ± 1.12 g) and multiple stresses (11.19 ± 2.61 g) when compared to the control plants (24.85 ± 2.87 g). The mutant *rpp13* (drought, 19.19 ±1.36 g; multiple, 16.11 ± 1.66 g) and *c2h2zf* (drought, 17.61 ± 0.45 g; multiple, 18.86 ± 1.9 g) had significant increase in the FSW under drought ($p \leq 0.005$) and multiple stress ($p \leq 0.05$) when compared to the wild type plants (drought, 13.23 ± 1.12 g and multiple, 11.19 ± 2.61 g). The *per* mutant showed higher FSW under all treatments (control, 44.72 ± 8.54 g; drought, 18.85 ± 0.2 g, nematode, 44.57 ± 3.84 g and multiple, 22.82 ± 2.61 g) than the wild type plants (control, 24.85 ± 2.84 g; drought 13.32 ± 1.12 g, nematode, 41.46 ± 6.74 g and 11.19 ± 2.61 g). The *c2h2zf* (50.06 ± 6.35 g) mutants had higher FSW in the control conditions than the wild type plants ($p \leq 0.01$, 24.85 ± 2.84 g). The *shr5* mutant (19.8 ± 2.3 g) had lower FSW than the wild type plants (41.46 ± 6.74 g) under nematode stress treatment ($p \leq 0.05$). Under nematode stress, the wild type plants had higher FSW then the control plants ($p \leq 0.01$, 24.85 ± 2.87 g) (Figure 4.3.A).

The shoot dry weight (SDW) of wild type plants was significantly reduced under the drought (4.23 ± 0.63 g) and multiple stress treatment ($p \leq 0.05$, 4.07 ± 0.85 g) compared to the control plants (9.25 ± 1.61 g). In contrast, the mutants *per* and *rpp13* had increased SDW under the drought (*per*, 6.19 ± 0.21 g and *rpp13*, 6.27 ± 0.48 g) and multiple stress treatments (*per*, 7.55 ± 0.85 g and *rpp13*, 5.45 ± 1.05 g) when compared to the corresponding treatments of the wild type plants. The mutant *per* (15.02 ± 0.66 g) had significantly higher SDW and the mutant *shr5* (8.51 ± 1.58 g) had lower SDW than the wild type plants (9.52 ± 1.61 g) in the control conditions ($p \leq 0.05$) (Figure 4.3.B).

The fresh root weight (FRW) of wild type and mutants was significantly reduced under drought (wild type, 8.92 ± 0.84 g, *per*, 12.63 ± 1.15 g, *rpp13*, 13 ± 1 g and *c2h2zf*, 15.11 ± 0.35 g) and the multiple treatment (wild type, 10.42 ± 3.55 g, *per*, 16.77 ± 1.99 g, *rpp13*, 11.67 ±2.42 g and *c2h2zf*, 17.39± 1.17 g) when compared to the corresponding control plants (wild type 56.43 ± 6.27 g, *per*, 67.29 ± 11.9 g, *rpp13*, 39.45 ± 4.7 g and *c2h2zf*, 62.99 ± 1.26 g). The FRW of the *shr5* mutant was not affected by the stress treatments, and under the drought stress (15.84 ± 1.51 g) it was significantly higher than the corresponding wild type plants ($p \leq 0.001$, 8.92 ± 0.84 g) (Figure 4.3.C).

The nematode stress treatment had no significant influence on the growth parameters of the wild type and mutant Nipponbare plants. Whereas, the drought and the simultaneous multiple stress treatment negatively influenced growth parameters in the wild type, *rrp13* and *c2h2zf* mutants. The mutants *per* and *shr5* were generally less affected by the drought and multiple stress than the wild type plants.

4.3.2. Yield parameters

The number of inflorescences per plant in the wild type plants was reduced under the multiple stress treatment (2.37 ± 0.66) when compared to the control plants (9 ± 1.09, $p \leq 0.005$). The reduction was also seen in the mutants *rrp13* (2.33 ± 1.33) and *c2h2zf* (2 ± 0) ($p \leq 0.01$) when compared to the corresponding controls (*rpp13*, 11.66 ± 2.18 and *c2h2zf*, 14.5 ± 2.5). In contrast, the s*hr5* mutant (7.33 ± 1.33) had significantly more inflorescences than wild type plants (2.37 ± 0.66; $p \leq 0.01$) following multiple stress treatment. The *per* mutant had increased inflorescence number under all treatments, control (*per, 26.33* ± 5.2; WT, 9 ± 1.09 $p \leq 0.01$), nematode (*per*, 25.33; WT, 11.71 ± 1.37, $p \leq 0.005$) and multiple (*per* 8 ± 1.52; WT, 2.37 ± 0.66 , $p \leq 0.01$) compared to wild type plants under the same stress treatments (Figure 4.4.A).

All the mutants had a significant reduction in seeds produced per plant under the drought stress (WT, 0.59 ± 0.28; *per*, 0.35 ± 0.14; *rpp13*, 0.58 ± 0.30; *shr5* ± 0.19 and *c2h2zf*, 0.00) and multiple stress (WT, 0.32 ± 0.078; *per*, 0.77 ± 0.023 , *rpp13*, 0.19 ± 0.12; *shr5*, 2.57 ± 1.97 and *c2h2zf*, 0.00) treatment when compared to control plants (WT, 12.91 ± 1.51; *per*, 9.82 ± 1.55, *rpp13*, 8.56 ± 1.90; *shr5*, 8.55, 2.19 and *c2h2zf* 10.22 ± 0.46). The nematode infection did not affect seed production in wild type or mutant plants except for the *shr5* mutant (5.04 ± 0.75) that produced fewer seeds per plant compared to wild type (14.19 ± 2.16). Under multiple stress treatment, *per* (0.77 ± 0.02) produced more seeds ($p \leq 0.005$) than the corresponding wild type plants (0.32 ± 0.078) and *c2h2zf* produced no seeds (Figure 4.4.B).

The extend of chlorosis and leaf senescence was not systematically measured but visual observations show that the *shr* mutants suffered high degree of chlorosis and leaf senescence in control and all three stressed condition. The other three mutants showed reduced leaf senescence when compared to the wild type plants under corresponding

stress conditions. The *per* mutants retained green leaves with reduced leaf senescence in all conditions.

4.3.3. Nematode susceptibility

All the mutants supported significantly reduced nematode multiplication ($p \leq 0.05$) under the nematode (*per*, 56,428 ± 17,190; *rpp13*, 77,710 ± 28,863 and *shr5*, 15,448 ± 0) and multiple stress treatments (*per*, 10440 ± 9948.3; *rpp13*, 7050.2 ± 6710.2; *shr5*, 1989.7 ± 443.81 and *c2h2zf*, 2901 ± 1135.4) when compared to the wild type Nipponbare plants (WT, 6,75,132 ± 1,88,438). The *c2h2zf* mutants supported no nematode reproduction under well-watered conditions. (Figure 4.5).

4.4. Discussion

Transgenic plants that exhibit drought tolerance in laboratory experiments frequently fail to exhibit the same desired characteristics in the field. For inferring drought or disease tolerance in a transgenic plant it is essential to evaluate its performance under realistic conditions (Deikman, Petracek and Heard 2012). The tissue culture and hydroponic methods of evaluating drought tolerance subject plants to severe and unrealistic stresses that rarely occur in nature. In contrast, in nature the crop species are exposed to prolonged moderate drought conditions that lead to compromised growth and yield. Similarly, nematode infection in tissue culture represents an unrealistic scenario, as the rate of infection in fields is influenced by the soil topology, moisture and plant health. The drought tolerance, nematode susceptibility and response towards the simultaneous multiple stresses of the rice mutants was evaluated in a realistic set-up and thus the severe drought stress assay to test survival efficiency after re-hydration was not performed. The mutants were subjected to the stress set up similar to that of the microarray experiment with focus on the plant performance rather than on just survival.

The four genes analysed in the study had significant expression changes under drought, nematode and simultaneous multiple-stressed plants compared to the control plants, determined by using one-way ANOVA followed by S-N-K posthoc test ($p \leq 0.05$). Two of the four proteins studied contained leucine rich repeat domains; the *SHR-5* gene encodes a protein with a LRR-receptor like domain and the disease resistance RPP13-like gene also encodes a R (resistance) protein LRR with a nucleotide binding site. Under normal growth conditions, the *shr-5* mutants show no difference in growth and yield parameters but have reduced fresh shoot weight and increased tiller number compared to wild type plants. Drought stress induces a bigger root system but does not affect other growth parameters of the *shr-5* plants. The simultaneous biotic and abiotic stress imposed on *shr-5* mutants increased the number of tillers and the number of inflorescences but the biomass and weight of seeds did not increase. The *SHR-5* gene encodes a receptor kinase-like protein, belonging to the leucine rich repeat-receptor like kinase family (Vinagre *et al.* 2006). The members of this receptor kinase family are broadly divided into two functional groups. The first group controls plant growth and development and the second group is involved in plant-pathogen interactions, plant-symbiont interactions and early stages of nodulation (Shiu *et al.* 2004). The LRR-LRK

are an important part of plant innate immunity, they recognise the pathogen-associated molecular patterns (PAMPs) and initiate down-stream signalling cascades involving MAPK. The members of LRR-RLKs, *Xa3*, *Xa21* and *Xa26* in rice confer resistance to the bacterial pathogen *Xanthomonas oryzae* (Song *et al.* 1995). The *OsBRR1* gene mediates resistance to the rice blast pathogen, *Magnaporthe oryzae* (Peng et al. 2009). We observed a low nematode reproduction in the *shr-5* plants under only nematode as well as the simultaneous dual stress. The results indicate that SHR-5 might be required for establishment of the RKN infection. In spite of reduced nematode number the nematode infestation reduced seed yield and biomass in the *shr-5* plants when compared to the nematode infected wild type plants. The sugarcane *SHR-5* gene is the closest relative of rice *SHR-5* gene; it shows a positive role in plant association with endophytic bacteria (Vinagre *et al.* 2006).

Under normal growth conditions, the loss-of-function mutation in the disease resistance *rpp-13* gene does not affect growth and yield parameters. Nematode infection does not affect growth or yield parameters of the *rpp-13* plants, but they support significantly lower nematode multiplication than the wild type Nipponbare plants. The results suggest RPP-13 like protein might have negative role in resistance against nematodes. Similar results are seen in sugarcane where RPP13-like proteins act as the negative regulator of resistance against red-rot disease (Gupta *et al.* 2010). The disease resistance RPP13-like protein is a member of the disease resistance *R* gene family. The *R* genes in plants initiate a hypersensitive response to pathogen attack and confer resistance to various pathogens. The proteins encoded by these genes have a nucleotide binding site (NBS) and a leucine rich repeat (LRR) that recognises the pathogen induced ligands (Meyers *et al.* 1999). There are 468 identified *R* genes in Nipponbare rice (Yang *et al.* 2006). The Arabidopsis RPP13-like protein is known for specific recognition of and imparting resistance towards *Peronospora parasitica* (downy mildew) (Bittner-Eddy *et al.* 1999; Bittner-Eddy and Beynon 2001). The mutants exhibit a drought tolerant phenotype with more biomass, bigger root system, higher number of tillers and short plant height when compared to the wild type Nipponbare plants but the yield parameters are unaffected. There is no previous report of the involvement of disease resistance RPP13-like proteins in abiotic stress. The drought tolerant phenotype of the *rpp-13* mutants is a unique observation and warrants further investigation.

The *c2h2zf* plants do not show deviation from the growth pattern observed in the wild type Nipponbare plants, expect for having more tillers. The rice genome has 189 C2H2 zinc finger genes, that are involved in all stages of reproductive development, from panicle initiation to seed maturation (Agarwal *et al.* 2007). Different members of the C2H2 zinc finger family impart tolerance to abiotic stress. C2H2-type zinc finger protein ZFP182 is required for ABA-induced antioxidant activity, whilst transgenic rice lines over-expressing ZFP245 and ZFP252 show enhanced cold, drought and salt tolerance (Huang *et al.* 2009a; Zhang *et al.* 2012). The *c2h2-zf* plants performed better than the wild type plants under the drought and multiple stresses with increased bio-mass, root mass and tiller numbers. The drought and salt tolerance (*DST*) gene, encodes a C2H2-zinc finger protein that acts as a negative regulator of drought and salt stress in rice. The *dst* mutants are less sensitive to drought stress when compared to the wild type plants, similar to the results obtained in our study for *c2h2-zf* plants. The *c2h2zf* mutants also have higher fresh weight than the wild type plants, retaining more water in a similar manner to the *dst* mutants (Huang *et al.* 2009b). The mutation in *DST* does not affect the agronomic and phenotypic growth parameters but the plants have wider leaves and reduced stomatal density (Huang *et al.* 2009b). Recent findings indicate that RNA interference of *DST* results in plants that produce more panicles and seeds than wild type plants. Thus, DST acts as a negative regulator of the reproductive meristem activity and reproduction (Li *et al.* 2013a). Interestingly, in our study, the loss-of-function mutation in the *C2H2-ZF* gene, drastically affected the yield parameters. No inflorescences were initiated under the drought stress and although under the multiple stress inflorescences were seen, no seeds were produced. Indicating that under the drought condition, C2H2-ZF proteins can positively regulate seed production in the plants. Although the *c2h2zf* plants showed reduced nematode susceptibility, no final conclusions could be made because there were no replicates available. Under simultaneous drought and nematode stress, the plants showed reduced nematode infection in comparison to the wild type plants under similar stress conditions.

The *per* mutants outperformed the wild type Nipponbare plants in growth parameters like tiller number, number of inflorescence and shoot weight in control conditions and under all three stress treatments. The *per* mutants produced more panicles in control, nematode stressed and simultaneous multiple stress treatment but significant yield gain was observed only under multiple stress condition. Peroxidases are heme-containing

proteins encoded by multigenic families and catalyse the oxidoreduction of various substrates using hydrogen peroxide (Passardi *et al.* 2004). They are involved in a broad range of physiological processes like auxin metabolism, lignin and suberin formation, cross-linking of cell wall components, defence against pathogens and cell elongation (Passardi *et al.* 2005). The peroxidases are usually induced after pathogen attack resulting in a primary oxidative burst (Torres, Jones and Dangl 2006). Interestingly, the peroxidase precursor in our study was down-regulated in all three treatment conditions in comparison to the control condition in roots as well as leaves. The T-DNA insertion mutants for the same gene show improved performance under the stress conditions, suggesting that it may act as a negative regulator of general stress. The peroxidase family in rice comprises 138 genes and 14 pseudogenes identified till now, however the genome is dynamic and is subject to constant rearrangements producing more copies of genes. This is the first record of a peroxidase acting as a negative regulator of a stress response. To confirm the negative role of this specific peroxidase in general plant stress responses it will be necessary to investigate the substrate for this peroxidase and the down-stream processes that might link with it.

These results indicate a role for each of these four genes in biotic, abiotic and simultaneous stress responses. Further investigation, including the hormone response of each mutant, analysis of over-expression lines and molecular characterisation of the interactions needs to be done to confirm their role in rice stress responses.

5 Chapter

The effect of simultaneous stress on Nipponbare and IR64 rice cultivars in field conditions

5.1. Introduction

Rice is the major cereal crop, which is the staple diet for half of the world population. By 2030, the demand for rice will increase by an estimated 2,000 million metric tons (FAO, 2002). The current climate prediction models suggest that the global climate change in coming years will result in variable rainfall and temperature leading to frequent droughts, floods and heat waves (Change 2007). In addition to this, the increase in temperature will lead to pathogen spread by influencing the habitat range of different pests and pathogens (Atkinson and Urwin 2012). In these changing climatic conditions, a wide range of concurrent environmental stresses will frequently challenge the rice crop. The increasing food demand and changing climatic conditions call for development of high performance rice varieties that are capable of producing higher yield under challenging environments. Use of traditional breeding programmes accompanied by molecular breeding and development of transgenic crops will be needed to attain the goal of providing crops with increased environmental tolerance. A stringent screening procedure both in controlled environment and in the fields has to be employed to ensure drought tolerance in these newly developed varieties (Deikman, Petracek and Heard 2012). The response to any type of stress is a complex interplay of various hormonal and signalling pathways. The molecular studies performed to investigate these pathways preferentially use the rice cultivar Nipponbare, a lowland japonica cultivar with a fully sequenced genome, in controlled environments but the popular commercial varieties like the lowland Indica cultivar IR64 has a diverse genetic make-up (Liu *et al.* 2007). Each variety's performance differs depending on the ecosystem it is grown in and the environmental factors acting on it. It is crucial to determine if the molecular responses elucidated using the preferred laboratory cultivar Nipponbare are also seen in the popular cultivars in their respective ecosystems. The knowledge obtained from molecular studies needs to be integrated with the breeding programs for developing improved high performance varieties.

5.1.1. Drought stress on the rice crop

Drought is a major limiting factor in rice production throughout the world and even mild drought stress can result in substantial yield losses (Guan *et al.* 2010). It affects 23 million hectares of rice producing area in South and South East Asia and 80 % of rainfed lowland rice in Africa (IRRI). By 2050, It is estimated that rice productivity will reduce by 14 % in South Asia, 10 % in East Asia and pacific, and 15 % in Sub-Saharan Africa due to water scarcity (Nelson 2009). As a strategy for efficient water management, rice is grown in aerobic conditions with a yield penalty. The plants grown in aerobic conditions show reduced leaf area index, biomass and 22 % yield reduction compared to the same cultivars grown in flooded conditions (Bouman *et al.* 2005). The effect of drought on yield and plant growth is dependent on the developmental stage challenged by drought. Drought during the vegetative phase results in reduced tiller numbers and causes half the yield penalty of drought experienced during panicle initiation and anthesis (Boonjung and Fukai 1996; Kamoshita *et al.* 2008). Similar results are supported by another study showing 21 %, 50 % and 21 % average yield loss due to drought during vegetative, reproductive and grain filling stages respectively (Sarvestani *et al.* 2008). Vegetative drought stress also leads to reduction of total biomass due to decrease in photosynthetic rate and accumulation of dry matter (Sarvestani *et al.* 2008). It has caused severe irreversible changes and yield loss in *Oryza sativa* cultivars of economic importance as well as the other genotypes (Efisue and Derera 2012; Zulkarnain *et al.* 2013). Other yield related parameters such as the plant height at maturation and total biomass accumulated reduce with drought stress at all developmental stages, especially in the vegetative stage (Boonjung and Fukai 1996; Guan *et al.* 2010).

5.1.2. Nematode infestation in the rice crop

Rice cultivars in different ecosystems are host to a wide range of PPN (Fortuner and Merny 1979). The rice root nematodes causing highest economic losses to rice cultivation include the migratory endoparasite *Hirschmanniella* spp. and the sedentary endoparasites *Heterodera* spp. and *Meloidogyne* spp. (Nicol *et al.* 2011). *Hirschmanniella* spp cause a yield loss of up to 25 %, *Heterodera sacchari* the most important cyst nematode on rice can cause yield loss of up to 50 % and the most important RKN species on rice, *M. graminicola* accounts for 17-30 % yield loss

(Bridge, Plowright and Peng 2005; Dutta, Ganguly and Gaur 2012). Once released into the soil, the egg masses of RKN species can survive in water logged soil for up to 14 months and the 2nd stage juveniles can start infecting when they have favourable aerobic conditions (Bridge, Plowright and Peng 2005). In upland and aerobic rice cultivation, the dry period initiates and accelerates infection by RKN such as *M. graminicola* and *M. incognita*. The dry aerobic phase in aerobic cultivation and vegetative drought stress in rainfed or irrigated systems provide an opportunity for the root nematodes to infect and establish a feeding site. Under these aerobic conditions, *M. graminicola* and *M. incognita* cause reduced dry-shoot biomass, reduced root length and stunted plants (De Waele *et al.* 2013; Jain, Khan and Kumar 2012; Greco *et al.* 2000). The sugarcane cyst nematode *H. sacchari* also infects rice in all ecosystems and causes chlorosis, reduced number of tillers and leaves under lowland as well as upland cultivation in West Africa (Babatola 1983; Coyne 2012).

5.1.3. Chlorophyll parameters as a measure of stress in plants

The environmental stresses experienced by a plant perturb various physiological processes thereby affecting plant performance. Chlorophyll concentration and chlorophyll fluorescence of the leaves are used to monitor photosynthetic performance of crop plants (Butler and Kitajima 1975). In contrast to the traditional method of measuring chlorophyll concentration by extraction, the Soil Plant Analysis Development (SPAD) unit, termed a chlorophyll meter, non-destructively measures the relative chlorophyll content of the leaves of crop plants (Kumagai, Araki and Kubota 2009; Netto *et al.* 2005). Measuring chlorophyll fluorescence can estimate energy transfer in the photosystems. In C4 plants, the activity of photosystem II shows a linear relationship with CO_2 fixation (Krall and Edwards 1992). Quantum efficiency of photosystem II is measured in terms *Fv/Fm* after a dark adaptation period and can be used as an estimate of plant photosynthesis, using the minimal fluorescence (F_0) maximal fluorescence (*Fm*) and variable fluorescence ($Fv = Fm - F_0$) (Butler and Kitajima 1975; Maxwell and Johnson 2000). The quantum efficiency of photosystem II in most of the measured plants reduces under stress due to photoinhibition (Maxwell and Johnson 2000). The quantum efficiency and the chlorophyll content both decrease in rice and other crop species under drought stress (Pirdashti, Sarvestani and Bahmanyar 2009; Li *et al.* 2006b; Chen *et al.* 2011a; Cha-Um, Yooyongwech and Supaibulwatana

2010). The similar effect is also seen when plants experience biotic stress. The planthopper *Nilaparvata lugens* feeding on rice leaves leads to decreased relative chlorophyll content (SPAD readings) and also slows down the rate of photosynthesis (Watanabe and Kitagawa 2000). Infection with the root-knot nematode *M. javanica* causes reduction in chlorophyll content of tomato and mung beans as measured by traditional chlorophyll extraction methods (Loveys and Bird 1973; Ahmed *et al.* 2009). *M. graminicola* and *Helicotylenchus multicinctus* also reduce chlorophyll content of rice plants (Dutta, Nayak and Prasad 1990; Mishra and Mohanty 2008) A reduction in photosynthetic rate measured as maximum quantum yield (*Fv/Fm*) and relative chlorophyll content (SPAD readings) is seen in rice plants infected with the cyst nematode *Heterodera sacchari* (Audebert *et al.* 2000; Blouin *et al.* 2005).

5.1.4. Hormonal cross talk between biotic and abiotic stress responses

The plant response to a combination of stresses encountered in the natural environment is regulated by overlapping suites of genes. The differential expression of these suites of genes dictates the hormonal and signalling pathways that trigger the tailored response for combinations of various stresses. Abscisic acid (ABA) is the major abiotic stress responsive phytohormone involved in response to drought, osmotic and salt stress, cold and high temperature specific response. The phytohormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) control the plant's induced defence response during pathogen attack (Nahar *et al.* 2011; Pieterse *et al.* 2009). SA is usually associated with biotrophic pathogens whereas JA and ET are associated with wounding responses caused by necrotic pathogens and herbivore attack (Nahar *et al.* 2011). Elevation in systemic and local levels of SA leads to induction of systemic acquired resistance, initiating an immune response in uninfected plant parts (Durrant and Dong 2004). The SA-mediated induced systemic resistance leads to activation of pathogenesis-related proteins especially the PR1b gene that is widely accepted as the marker of SAR (Vlot, Dempsey and Klessig 2009). The SA independent SAR shows activation of a JA activated myb gene called *OsJAmyb* that is induced in a susceptible pathogen interaction (Lee, Qi and Yang 2001). JA plays an important role in plant defence against RKN infection and in roots the JA pathway works in conjunction with the systemic induction of ET (Nahar *et al.* 2011). JA and ET act synergistically to each other and both are required for induction of a defence response but they are known to act mutually

antagonistically to SA signalling (Kunkel and Brooks 2002). The biotic stress responsive hormonal signalling pathways are negatively influence by ABA signalling (Mauch-Mani and Mauch 2005). In rice, ABA interferes negatively with the SA, ET and JA response pathways and reduces plant defence toward nematodes (Nahar *et al.* 2012). The cross talk between the biotic and abiotic stress pathways is characterised by differential regulation of various transcription factors (Singh, Foley and Oñate-Sánchez 2002). A plant-specific transcription factor family, WRKY, that has 98 members in japonica rice cultivars and 102 in indica cultivars, is involved with abiotic stress responses, biotic stress response and in fine tuning the crosstalk between both (Ross, Liu and Shen 2007). The hormonal and signalling pathways have been elucidated by molecular laboratory studies by applying one or more stresses to rice plants, exogenous application of hormones or by reverse genetics. The evidence for similar molecular responses in the field is scarce.

We have evaluated performance and molecular response of lowland rice cultivars under drought stress, nematode stress and simultaneous drought and nematode stress in the field. The stress treatments have been carefully optimised to mimic the natural vegetative drought stress experienced by rice under aerobic conditions and the level of nematode infestation seen in the rice field.

5.2. Materials and methods

The field study was conducted at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria in the dry season (December - March), with help of Dr Danny Coyne and with the help of Africa Rice Unit, IITA. Two lowland rice cultivars, IR64 (Indica) and Nipponbare (Japonica) were challenged by drought, RKN infection and simultaneous drought and nematode infection (RKN – *Meloidogyne incognita* and CN - *Heterodera sacchari*).

5.2.1. Field preparation

The field was divided into five individual plots 3.6 m * 6.5 m (Figure 5.1A). Plot 1 was previously under rice cultivation to increase the density of the cyst nematode *Heterodera sacchari*. Plots 2 and 4 had been cultivated with *Celosia argentea* and *Zea mays* for increasing the density of RKN *Meloidogyne incognita.* These three plots were left fallow for three weeks before the rice seedlings were transplanted into the soil. Plots 3 and 5 were left uncultivated for three weeks after nematicide treatment. For nematicide treatment, the granular organophosphate cadusafos (Rugby 10G™) was applied twice over a period of two weeks at a rate of 1.3 g/m^2 (Figure 5.1B). A 1 m deep and 0.5 m wide trench separated drought stressed plots (1-3) from the watered plots (4-5) (Figure 5.1A). Each plot was divided into 30 sub-plots (42 x 80 cm) (Figure 5.2). During the fallow period, all the plots were watered each day. The whole field had a high density of the stubborn weed grass *Cyperus esculentus,* yellow nutsedge. Manual weeding was performed regularly after transplantation of rice and throughout the stress regimes. The fields were fertilised once before transplantation and again one month after transplanting the trial using N.P.K 15:15:15, 400 kg/ha.

5.2.2. Experimental design

Figure 5-1A gives a schematic representation of the plots and treatment assigned to each plot. Each treatment plot was divided into 30 equal-sized sub-plots (Figure 5-2A and B). Nipponbare and IR64 seedlings were transplanted in alternate sub-plots. The seeds of both cultivars were first germinated in 72 cell trays in a net-house, three weeks prior to transplantation (Figure 5.3A). Fifteen seedlings per treatment of each cultivar were

manually transplanted in each of their designated sub-plots (Figure 5-2A and Figure 5-3B-C). Two, 1 m long Sentek pipes were installed in each treatment plot, one in each cultivar-specific sub-plot, for soil moisture measurements (Figure 5-4A). Each pipe was installed in the centre of a sub-plot, surrounded by four seedlings (Figure 5-4B). The 1 m long Diviner 2000 soil moisture probe that measures volumetric soil moisture content (Sentek Sensor Technologies, Stepney SA, Australia) and data logger were used for soil moisture profiling of 1 m of soil twice each day (Figure 5-4C).

5.2.3. Data collection

Three 100 g soil samples were collected from different parts of each treatment plot just before transplantation of seedlings. The 100 g of soil from each sample was diluted to make a total volume of 100 ml for each soil sample and the plant parasitic nematodes present were counted and morphologically identified. The nematode density in each plot was determined by taking a mean of six counts (two counts / sample / plot) (Table 21).

All plots were watered twice a day, at 10 am and 4 pm; 20 litres of water was given to each sub-plot at each occasion. Soil moisture was recorded twice a day before each watering session. Nine plants at random were marked in each sub-plot for morphological measurements to be taken at time of harvest. Chlorophyll content was measured as SPAD values on one fully expanded leaf of six plants per sub-plot (picked at random) Photosystem II dark reaction adaptation readings were taken at 4'o clock each day. The leaves were dark adapted for 20 min and the readings were taken on the abaxial side of a fully expanded leaf for each of three plants (picked at random) per sub-plot.

Watering was withheld from plots 1, 2 and 3 at two weeks after transplantation. Soil moisture was recorded twice each day to monitor severity of the drought stress. Leaf rolling scores were used to indicate the level of drought stress being experienced by the plants.

After two weeks of severe vegetative drought stress leaf tissue samples were collected for molecular study. A fully expanded leaf, other than the flag leaf, was collected from each plant. The tissue was preserved in RNAsave (Biological Industry Israel Beit-Haemek Ltd, Israel) according to the manufacturer's instructions. The samples were stored in RNAsave at 4 ºC before transportation. Within one month from the day of

sampling, the samples were transported to the University of Leeds and total RNA was extracted.

The drought stressed plants were watered once a day after the sample collection and until harvest in order to maintain an intermediate level of drought stress. The nematode stressed plants and control plants continued to receive water twice a day. All plants were harvested 2 months after transplantation to the field. Biomass, morphological parameters and yield parameters of three marked plants in each sub-plot were measured at time of harvest.

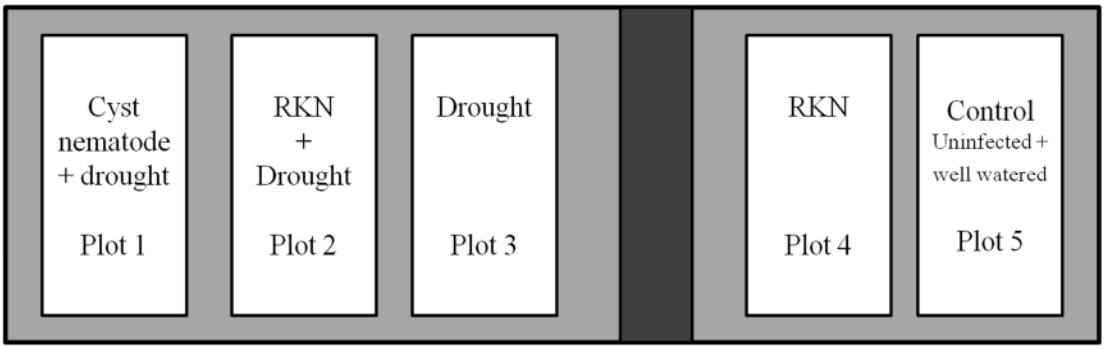
5.2.4. Expression analysis of stress responsive genes

RNA extractions were performed on three replicate samples for each cultivar per treatment type. Each replicate consisted of pooled leaf tissue from four individual plants using those samples that were best preserved upon arrival in Leeds. An equal quantity of total RNA from each replicate was pooled to make 1 µg of RNA to be used for cDNA synthesis (Section 2.2.16.2). Expression analysis of stress responsive genes was performed by qRT-PCR using a 5-fold dilution of 1st strand cDNA, the primers detailed in Table 22 and the reaction conditions as described in Table 4-5.

5.2.5. Statistical analysis

Data analysis was performed using the IBM SPSS statistic 19 package. The nature of the data obtained was explored using the descriptive statistic function by conducting normality tests and studying histograms. No data obtained had a Normal distribution. Thus, the non-parametric Mann-Whitney U test was performed to compare each treatment to the control plants. The significance difference was determined at $p \leq 0.05$

A.



B.

Plot	Previously cultivated	Nematode infestation	Fallow period	Watering
Plot 1	*Oryza sativa*	*H. sacchari*	3 weeks	Well watered
Plot 2	*Celosia argentea*	*M. incognita*	3 weeks	Well watered
Plot 3	*Celosia argentea*	Treated with Nematicide	3 weeks	Well watered
Plot 4	*Celosia argentea + Zea mays*	*M. incognita*	3 weeks	Well watered
Plot 5	*Zea mays*	Treated with Nematicide	3 weeks	Well watered

Figure 5-1. The field set-up for studying the effect of drought, nematode infection and simultaneous drought and nematode infection on rice in the field.

A. The field was divided in five equal sized plots (3.6 m * 6.55 m). The treatments were assigned to each plot depending on the previous cultivation and nematode population in the soil. The first three plots were drought stressed and were separated from the other two by a 1 m deep trench, to avoid water sweeping through the soil.
B. Detail of cultivation and plot preparation prior to transplantation of rice seedlings.

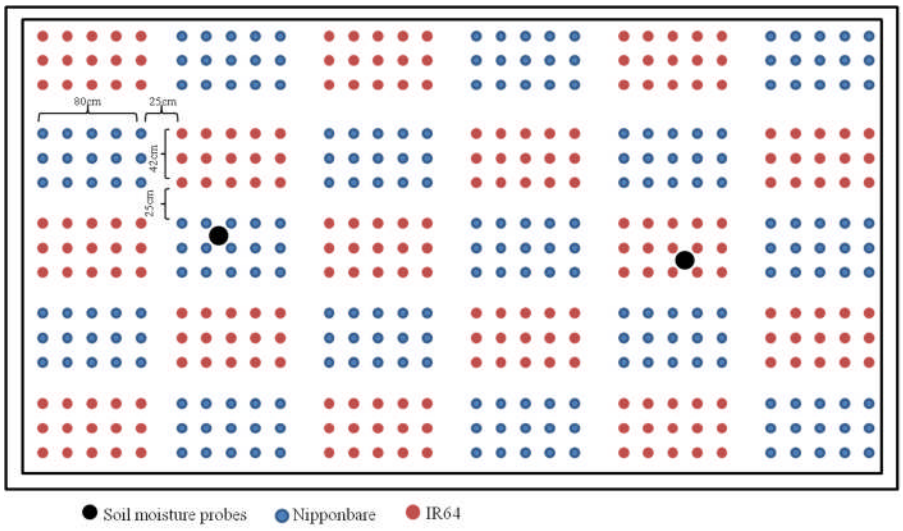


Figure 5-2. Standard outline of a treatment plot

Each treatment plot has 30 sub-plots, fifteen for each of the two rice cultivars Nipponbare and IR64. Each sub-plot was planted with fifteen seedlings.

A. Schematic representation of each treatment plot. The blue dots represent the Nipponbare seedlings and the red dots represent the IR64 seedlings; the black dots represent the soil moisture probes installed. Each treatment plot was bordered with two rows of unstressed rice plants.
B. A treatment plot with markings before transplantation.

Figure 5-3. Seedling nursery and transplantation to the field

A. The seeds were germinated in 72 cell trays and for first three weeks they were grown in a net house.
B. After three weeks the seedling of each cultivar were individually manually transplanted into the designated sub-plots
C. A treatment plot after transplantation of three-week-old seedlings.

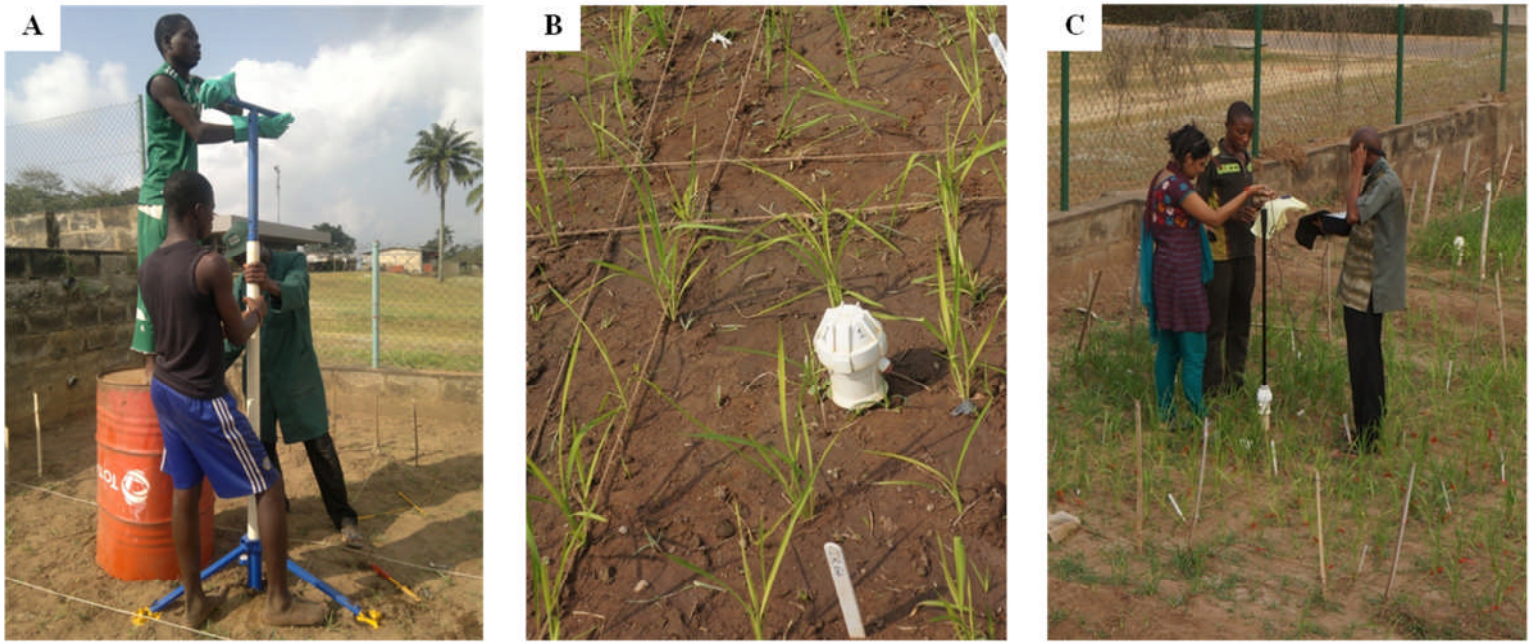


Figure 5-4.Installation of Divina soil moisture probes for profiling soil moisture up to the depth of 1 metre.

A. One metre long Sentex pipes were installed in sub-plots. Care was taken to have air-tight installation.
B. The tops of installed pipes were tightly secured with lids to prevent moisture and impurities (like soil and dust particles) from interfering with the soil moisture profiling.
C. Soil moisture was recorded twice each day, using the one metre-long soil moisture probe.

Plot number	Treatment	Initial nematode density / 100 g of soil
Plot 1	Cyst nematode and drought	343 cysts *H. sacchari* or 68,600 juveniles (considering each cyst has minimum of 200 eggs)
Plot 2	RKN and drought	57.5 RKN juveniles
Plot 3	Drought	2.5 – *Helicotylenchus*
Plot 4	RKN	25 RKN 10 *Helicotylenchus* 2.5 *Criconemella*
Plot 5	Control, uninfected	35 *Pratylenchus*

Table 21 Nematode densities in treatment plots before transplantation after nematicide treatments

Three replicate soil samples from different parts of each treatment plot were collected. 100 g of soil was diluted to a total volume of 100 ml for each sample and the nematodes present were morphologically identified and counted. A mean of total six counts (two / sample) was recorded as the initial nematode density of the field.

Gene name	Locus	Forward primer	Reverse primer
OsJAmyb	**LOC_Os11g45740**	GAGGACCAGAGTGCAAAAGC	CATGGCATCCTTGAACCTCT
WRKY 82	**LOC_Os05g14370**	CCAGCACAATGACCTCAACATCA	ACTGCCTTCTCACCACCGATT
ABI2	**LOC_Os01g40094**	TTGGTGTTCTTGCCATGTCG	TTCGTCATCCTTTGCTCGAG
DREB2A	**LOC_Os01g07120**	GCTGCACATCAGCACCTTCA	TCCTGCACCTCAGGGACTAC
PR1b	**LOC_Os01g28450**	GCCATGGCACTCCCCTCCCA	CTGCACGCTCGTGTCCCAGG

Table 22 List of primers used for quantitative expression analysis of gene involved in hormonal signalling pathways.

5.3. Results

The drought treatment was initiated two weeks after transplantation. Plots 1, 2 and 3 were drought-stressed whereas Plots 4 and 5 remained well watered (Figure 5.5 A-E). Meteorological data was obtained from the weather station at IITA, Ibadan. During the period of drought the average evaporation was 4.32 ± 0.18 mm, wind speed was 2.95 ± 0.09 km/h, min daily temperature was 18.85 ± 0.39 ºC, max temp was 33.40 ± 0.26 ºC, minimum relative humidity 20.25 ± 1.63 % and maximum humidity 89.18 ± 1.10 %.

5.3.1. Quantifying severity of drought imposed

The soil moisture was recorded for one sub-plot of each cultivar type in each treatment plot. Figure 5.6 A-D represents the soil moisture log for 15 days of drought stress in all treatment sub-plots with cultivar IR64 at various depths (A-5 cm, B-15cm, C-25cm and D-35cm). After four days of drought stress, the top layer of soil started to dry out in the drought stressed plots. In the cyst nematode and drought stressed plot (CND) the soil moisture in the top layer dropped from 9.39 ± 0.01 % to 5.02 ± 0.05 %, in the RKN and drought stressed plot (RKND) it dropped from 18.80 ± 0.23 % to 7.6 ± 0.07 % and in the drought stressed plot (D) it dropped from 14.25 ± 0.02 % to 1.93 ± 0.38 % (Figure 5.6 A). After 15 days, the soil moisture in the drought stressed plot was reduced to less than 25 % of the initial value. The soil moisture in the simultaneous drought and RKN stressed treatment plot decreased to 50 % of initial. Soil moisture content of the RKN-infected plot (RKN) and the control plot increased over the period of time (RKN – 11.47 ± 0.02 % to 12.05 ± 0.38 % and–C - 17.16 ± 0.02 % to 18.45 ± 0.40 % (Figure 5.6 B). At the depth of 15 cm the soil moisture in the control and cyst nematode plus drought stressed plots, did not fluctuate much during the drought stress period –C - 26.8 ± 0.18 % to 27.78 ± 0.41 % and CND - 9.33 ±0.02 % to 10.02 ± 0.11 %). The soil moisture in RKN and drought plot decreases from 18.23 ± 0 % to 12.39 ± 0.12 %, in the drought stressed plot it decreased from 21.67 ± 0.21 % to 9.66 ± 0.07 %. In the RKN infected plot the soil moisture increased from 16.87 ± 0.06 % to 22.21 ± 0.28 %. At 25 cm of soil depth, the soil moisture content of all treatments apart from drought and cyst nematode infected plot showed less fluctuation. The simultaneous drought stressed and cyst nematode infected plots had a drop in soil moisture content after two days of

drought and there after it stayed constant (Figure 5-6 C). At 35 cm depth, the RKN infected plot showed increase in soil water content over the period. The soil moisture in control plants stayed constant through for the 15 days. The soil moisture level in drought stressed plots beyond 25 cm depth was low than the control plants but constant throughout the treatment (Figure 5-6 D). The figure 5.7 A-D shows the soil moisture log for 15 days of drought stress in all treatment sub-plots with cultivar Nipponbare at various depths (A-5 cm, B-15cm, C-25cm and D-35cm). The soil moisture content at soil surface of drought stressed plots reduces to half of initial after 15 days of drought stress. In the CND plot it reduced from 9.12 ± 0.1 % to 5.38 ± 0.19 %, in RKND plot is reduced from 18.65 ± 0.06 % to 10.31 ± 0.04 % and in drought stressed plot it reduced from 15.55 ± 0.060 % to 6.96 ± 0.17 % (Figure 5-7 A). The soil moisture content of these three plots was lower than the control and RKN infected plants where it increased over the period of treatment (C - 21.07 ± 0.11 % and in RKN - 10.95 ± 0.29 % to 14.59 ± 0.20 %) (Figure 5-7A). At 15 cm of depth, the soil moisture content of the CND plot reduced from 11.55 ± 0.015 % to 8.03 ± 0.12 %, the RKND plot it decreased from 23.56 ± 0.12 % to 17.62 ± 0.08 % and in drought stressed plot it reduced from 15.98 ± 0.14 % to 11.58 ± 0.18 % (Figure 5-7B). The soil moisture content of RKN infected plants increased from 17.89 ± 0 % to 20.79 ± 0.07 % (Figure 5-7B). At 25 cm and 35 cm depth, the soil moisture in all treatments did not vary much throughout the treatment; the soil moisture content in drought stressed plots was lesser than the control and RKN infected plots (Figure 5-7C).

5.3.2. Effect of stress treatments on chlorophyll content and maximum quantum efficiency of photosystem II

The change in chlorophyll content of the plants was recorded during the period of drought treatment. Figure 5-8A shows the change in chlorophyll content of IR64 plants over the period of drought stress. Before induction of drought stress, chlorophyll content was lowest in the IR 64 plants infected with cyst nematodes (23.32 ± 2.00) SPAD unit and continued to be lowest in these plants throughout the drought stress regime. The mean chlorophyll content for the period in both sets of the simultaneous drought and nematode stressed plants was significantly lower than for the control plants (CND -23.41 ± 0.72 SPAD unit , RKND - 27.9.29 ± 0.68 SPAD unit and control plants

29.77 ± 0.40 SPAD unit)(Figure 5-8 B). The chlorophyll content of Nipponbare plants showed a trend similar to the IR64 plants (Figure 5-9 A). The cyst nematode infected and drought stressed plants had the lowest chlorophyll content (19.82 ± 3.02 SPAD unit) both at the beginning of the experiment and throughout the treatment. The plants simultaneously stressed with nematodes and drought showed reduced mean chlorophyll content (CND -22.56 ± 1.42 SPAD unit, RKND – 28.00 ± 0.52 SPAD unit and control plants 29.93 ± 0.69 SPAD unit) (Figure 5-9 B).

During the stress period the photosynthetic activity of photosystem II was measured as maximum quantum yield after dark adaptation. Table 23 shows the maximum quantum yield at two time points during the drought stress treatment. i.e. 4 days post drought induction and 16 days post drought induction. Maximum quantum yield of IR64 plants was significantly reduced in all four treatments over the period of drought stress. Only drought and simultaneous drought and RKN infection caused a significant reduction in maximum quantum yield in the Nipponbare plants over the same period (shaded cells in Table 5-3). Plants under each treatment condition for both the cultivars were also compared to the unstressed controls at each of the two time points. Four days post induction of drought stress, the IR64 plants under drought and RKN infection already had a significantly lower maximum quantum yield. At 16 days post drought induction, IR64 plants under all stress treatments except drought alone had significantly lower maximum quantum yield than the control plants. The Nipponbare plants under drought stress and simultaneous drought and RKN infection had significantly reduced maximum quantum yield after 16 days of drought stress.

5.3.3. Hormonal signalling during stress treatments in the field

One fully expanded leaf from three plants of each sub-plot in each treatment type was collected for expression analysis of markers of hormonal signalling in field stress conditions. The leaf rolling score of the plants receiving a drought stress as part of their treatment was recorded weekly as a visible marker of drought stress. The tissue samples were collected only after 80 % of plants in each treatment had a score higher than five with a mean score also higher than five (Figure 5.10). The leaf rolling score of IR64 plants was higher than for Nipponbare plants under each corresponding treatment.

The expression of *PR1b* transcript increased in the Nipponbare plants simultaneously stressed with cyst nematode and drought and in plants only infected with RKN. The

drought stressed plants showed significant reduction in expression of *PR1b* compared to the control plants (Figure 5.11 A). The *ABI2* transcript was significantly up-regulated in all treatments that included drought stress and was down-regulated in the RKN infected plants. The expression of *DREB2* was significantly reduced in simultaneously cyst-nematode and drought stressed plants and in RKN infected plants. The transcript was abundant in simultaneous drought and RKN treatment and in only drought stressed plants. Expression of *OsJAMYC* was significantly higher in all four treatments than the control plants. Similarly, *WRKY82* was also up-regulated in all four treatments (Figure 5.11 B).

5.3.4. Effect of stress regimes on growth and yield parameters

The plants were harvested at the beginning of March 2013. At harvesting, a range of growth parameters – fresh shoot weight, dry shoot weight, fresh root weight and seed weight per plant were recorded for both cultivars under each stress treatment. The fresh shoot weight (FSW) of both cultivars was significantly reduced under all treatments that included a drought stress. FSW of IR64 under CND treatment was 21 % reduced (26.48 ± 0.71), in the RKND plot it was 30 % reduced (23.85 ± 0.70) and in drought treated plants it was 29 % reduced (23.585 ± 1.04) compared to the FSW of control plants (33.79 ± 1.08). FSW of Nipponbare under CND treatment was reduced by 26 % (21.60 ± 0.64), in RKND stress it was reduced by 35% (18.72 ±0.54) and under drought stress it was reduced by 44 % (16.16 ± 0.60) compared to the FSW of control plants (29.22 ± 0.65). Similarly, the dry shoot weight (DSW) of both cultivars was also significantly reduced under all treatments that included a drought stress. DSW of IR64 under CND treatment showed 27.7 % (14.06 ± 0.39), in RKND treatment it was reduced by 24.4 % (14.64 ± 0.45) and under drought stress it was reduced by 14.7 % (16.645 ± 0.79) but the RKN treatment caused a 20 % increase (23.35 ± 0.46) compared to the DSW of control plants (19.47 ± 0.56). DSW of Nipponbare under CND treatment was reduced by 33.5 % (12.36 ± 0.39), in RKND treatment it was reduced by 42 % (10.78 ± 0.34) and in drought stressed treatment it was reduced by 41 % (10.89 ± 0.42) compared to the DSW of control plants (29.22 ± 1.12). The fresh root weight (FRW) of both cultivars also decreased significantly under the drought stress treatments. The FRW of IR64 plants under the CND treatment was reduced 13 % (11.08 ± 0.21), in RKND stress it was reduced by 7 % (11.85 ± 0.28) and under drought stress treatment it

was reduced by 16 % (10.65 ± 0.37) but under only RKN infection it was increased by 8 % (13.22 ± 0.25) as compared to the FRW of control plants (12.79 ± 0.34). The FRW of Nipponbare plants under CND stress was reduced by 8 % (10.76 ± 0.19), in RKND stress it was reduced by 13 % (10.14 ± 0.19) and under drought stress it was reduced by 16.7 % (9.75 ± 0.24) compared to the FRW of control plants (11.69 ± 0.52). Drought stress treatments had a similar effect on the seeds produced per plant in both cultivars. The seeds produced per IR64 plant under CDN stress was reduced by 25.8 % (5.50 ± 0.18), in RKND stress it was reduced by 57 % (3.19 ± 0.10) and under only drought stress it was reduced by 54.8 % (3.34 ± 0.18) compared to the seeds produced by well watered and uninfected IR64 plants (7.47 ± 0.17). The seeds produced per Nipponbare plant under CND stress was reduced by 37 % (3.23 ± 0.11), in RKND stress it was reduced by 54 % (2.33 ± 0.06) and under only drought stress it was reduced by 65 % (1.78 ± 0.08) compared to well watered and uninfected Nipponbare plants (5.13 ± 0.141).

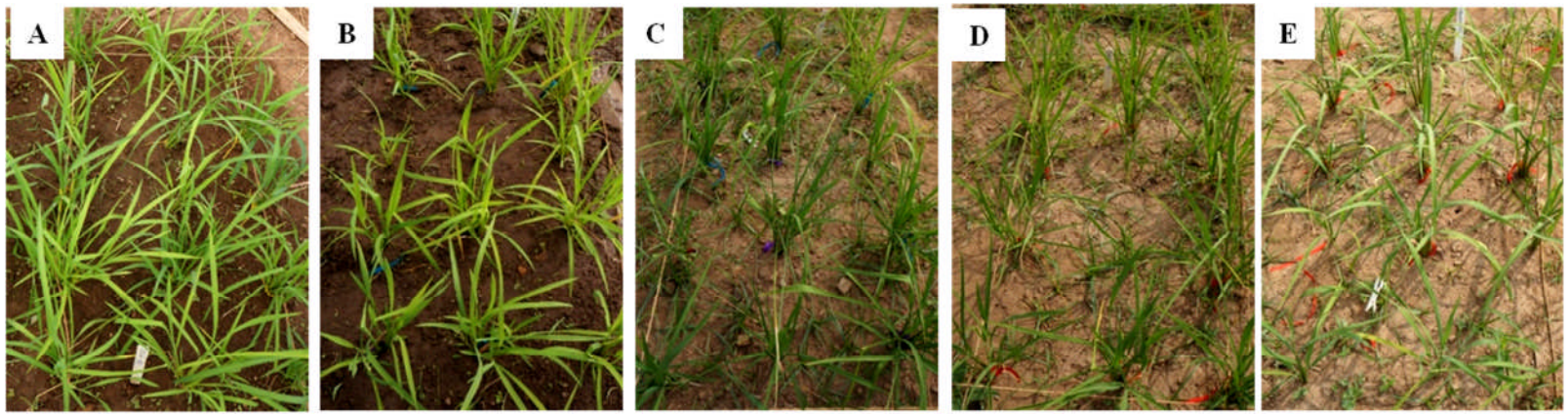


Figure 5-5 . Representative sub-plots from each treatment plot after seven days of drought stress

A. Control sub-plot, well watered and uninfected
B. Root-knot nematode infected sub-plot
C. Drought stressed and uninfected sub-plot
D. Drought stressed and RKN infected sub-plot
E. Drought stressed and cyst nematode infected sub-plot.

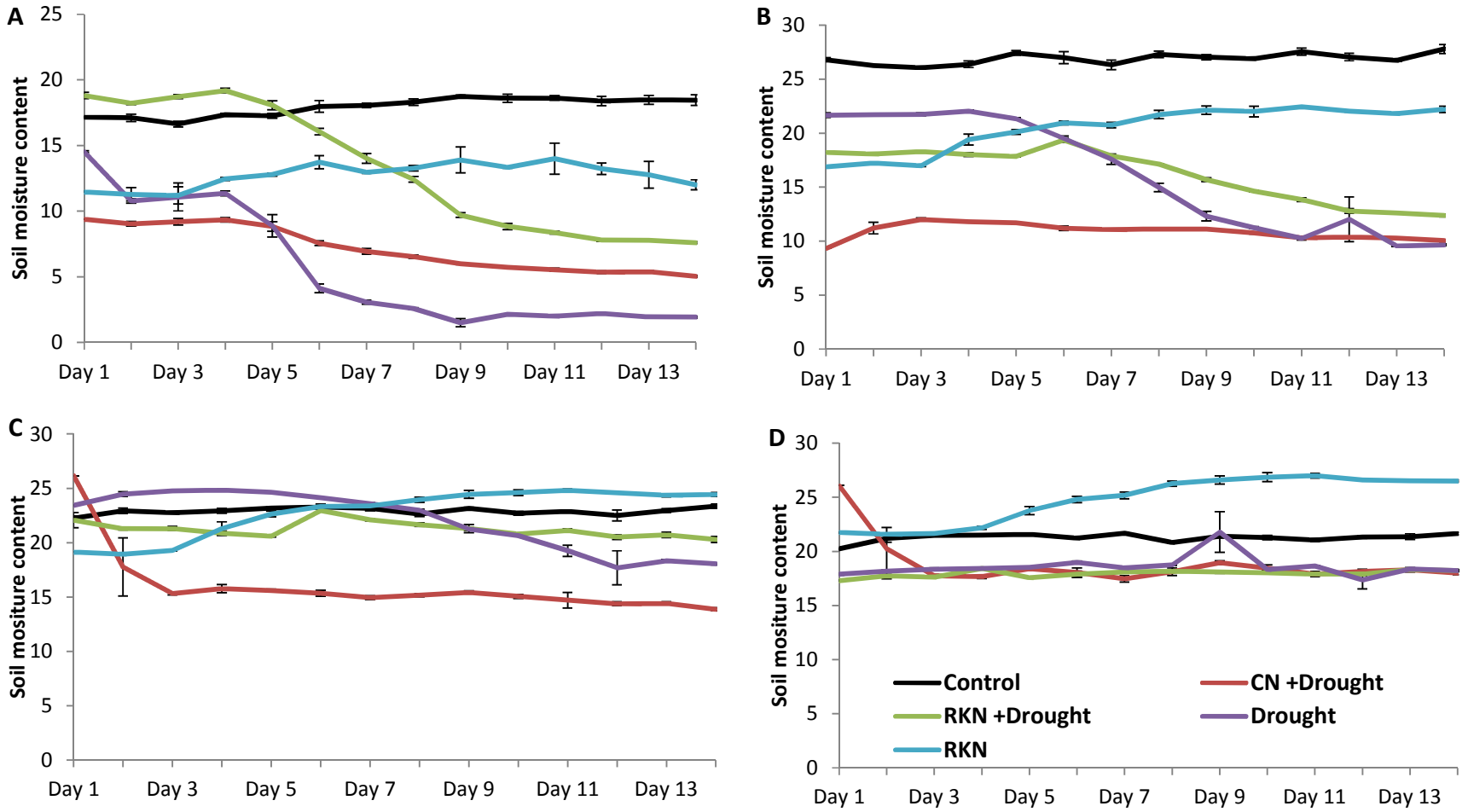


Figure 5-6. Volumetric oil moisture profile of each treatment plot over the period of drought stress for different soil depths for sub-plots with IR64 plants

The soil moisture was measured twice a day in each treatment. Each data point is a mean of four readings taken at different soil depths and the error bars denote standard error of mean. A-at 5 cm, B-at 15 cm, C-at 25 cm and D-at 35 cm from the soil surface.

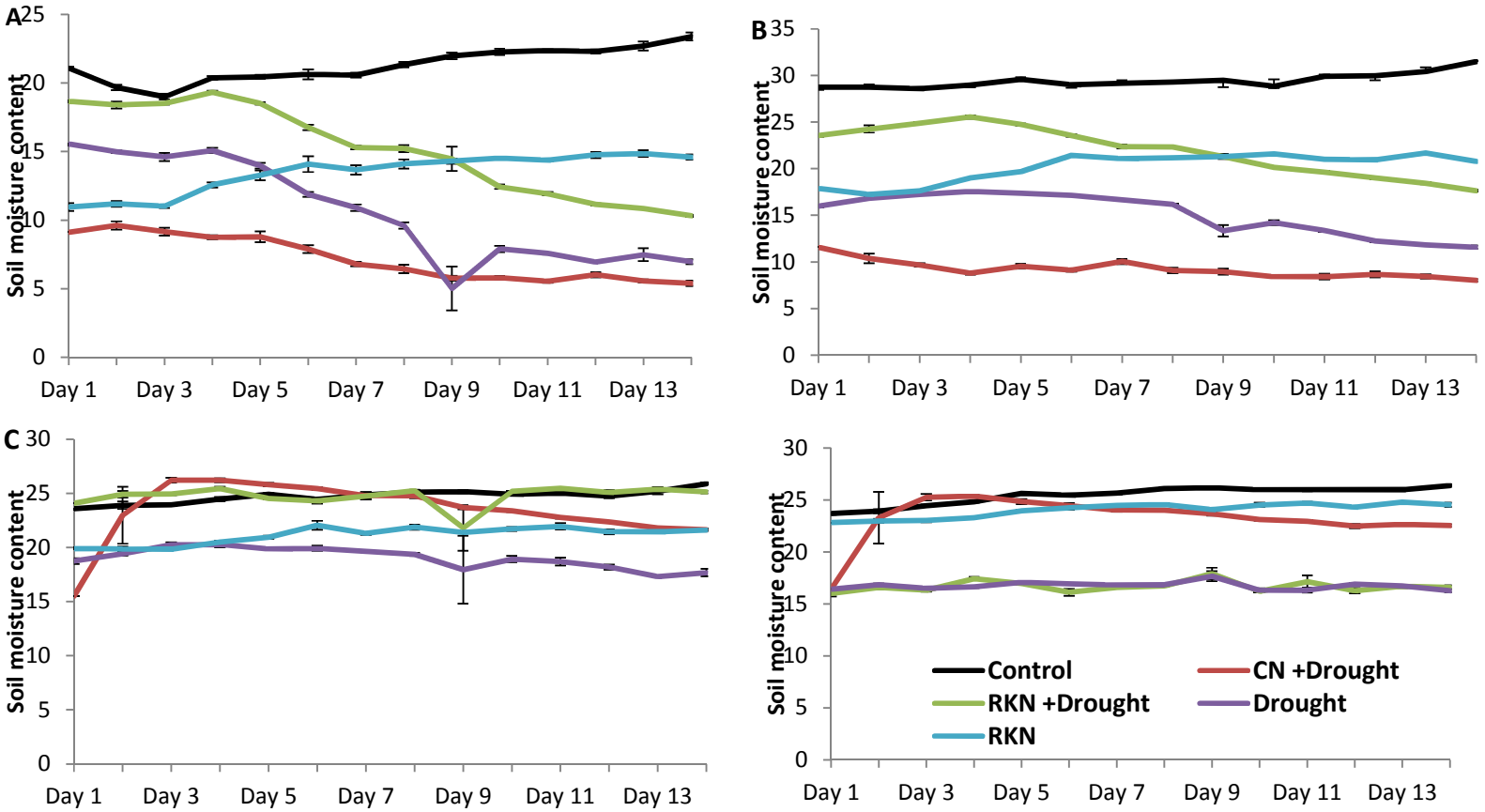


Figure 5-7- Soil moisture profile of each treatment plot over the period of drought stress for different soil depths for sub-plots with Nipponbare plants

The soil moisture was measured twice a day in each treatment. Each data point is a mean of four readings taken at different soil depths and the error bars denote standard error of mean. A-at 5 cm, B-at 15 cm, C-at 25 cm and D-at 35 cm from the surface.

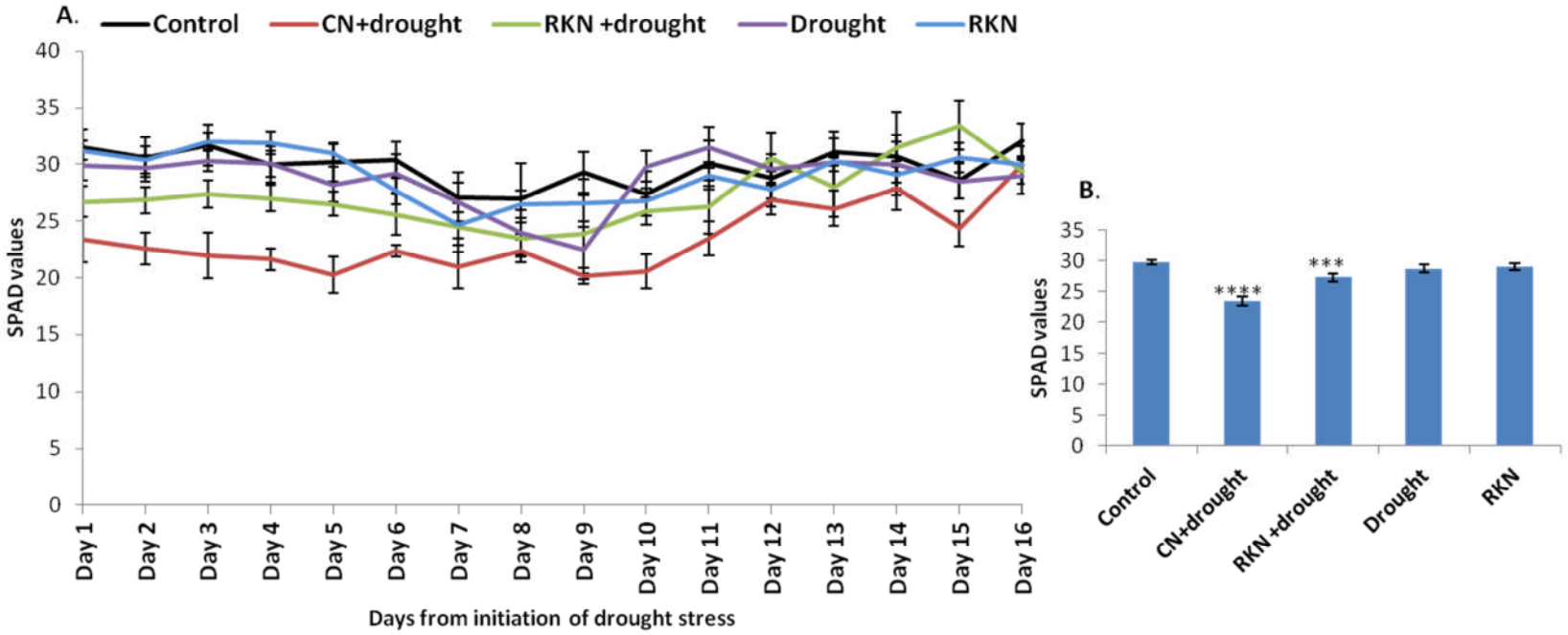


Figure 5-8. The chlorophyll content in leaves of IR64 plants under various stress treatments represented as SPAD values.

The chlorophyll content was measured as a mean of SPAD values from one fully expanded leaf from each of fifteen plants, from five sub-plots of IR64 across each treatment plot.

A. SPAD values for treatment type over the period of drought stress. Each data point is a mean of 75 SPAD readings from 75 fully expanded leaves (15 plants x 5 sub-plots) for each stress treatment.

B. Mean SPAD values for the duration of each treatment. Significance was analysed by Mann-Whitney U non-parametric test. at (****$P \leq 0.001$, *** $P \leq 0.005$).

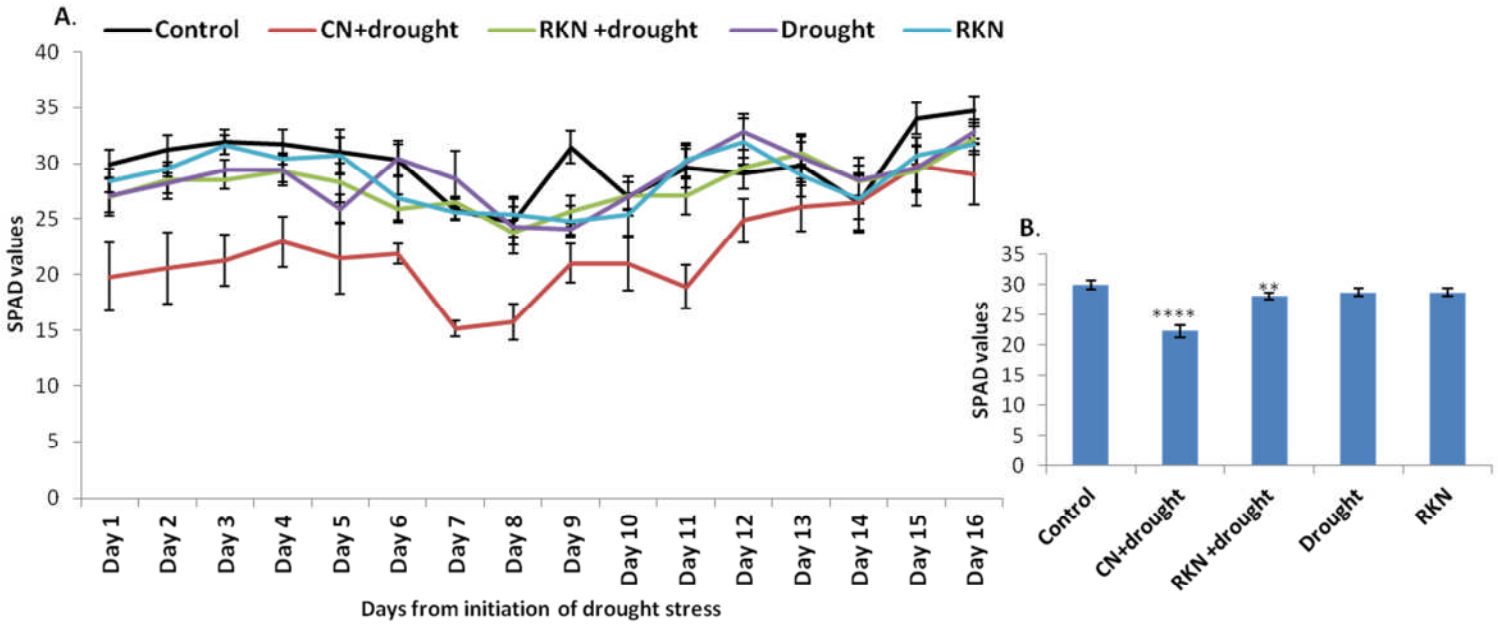


Figure 5-9. The chlorophyll content in leaves of Nipponbare plants under various stress treatments represented as SPAD values.

The chlorophyll content was measured as a mean of SPAD values from one fully expanded leaf from each of fifteen plants, from five sub-plots of Nipponbare across each treatment plot.

A. SPAD values for treatment type over the period of drought stress. Each data point is a mean of 75 SPAD readings from 75 fully expanded leaves (15 plants x 5 sub-plots) for each stress treatment.

B. Mean SPAD values for the duration of each treatment. Significance was analysed by Mann-Whitney U non-parametric test. at (****$P \leq 0.001$, *** $P \leq 0.005$).

Treatment	Cultivar	*Fv/Fm* after 4 days of drought stress	*Fv/Fm* after 16 days of drought stress
Control	IR64	0.73 ± 0.06	0.76 ± 0.08
	Nipponbare	0.77 ± 0.03	0.74 ± 0.03
Cyst nematode + drought	IR64	0.74 ± 0.06	0.49 ± 0.3*
	Nipponbare	0.71 ± 0.05	0.67 ± 0.05
RKN + drought	IR64	0.77 ± 0.02	0.70 ± 0.03*
	Nipponbare	0.77 ± 0.04	0.63 ± 0.02*
Drought	IR64	0.78 ± 0.04*	0.70 ± 0.02
	Nipponbare	0.78 ± 0.03	0.66 ± 0.04*
RKN	IR64	0.77 ± 0.02*	0.61 ± 0.08*
	Nipponbare	0.69 ± 0.1	0.66 ± 0.07

Table 23 Maximum quantum yield of photosystem PSII after dark adaption measured as *Fv/Fm* for IR64 and Nipponbare plants under stress treatments.

The Fv/Fm was measure on the abaxial side of a fully-expanded leaf, dark adapted for 20 minutes using dark adaption clips. The readings denote mean and standard deviation for ten readings for each treatment and cultivar type. The shaded cells denote final readings that differed significantly from the corresponding initial readings ($p \leq 0.05$). The treatments significantly different from the control for each cultivar at time of initial reading and/or final reading are marked with * ($p \leq 0.05$).

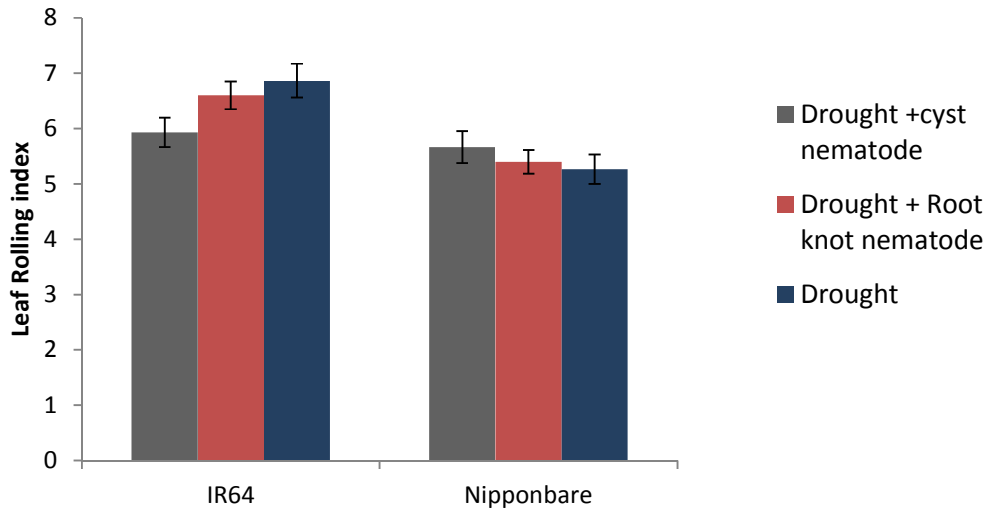


Figure 5-10. The leaf rolling score for IR64 and Nipponbare plants from drought stressed plots before collection of tissue sample

The leaves were scored every week after initiation of the drought stress. The tissue samples were collected after the leaves were rolled as a V, with a score of 5 and above for 80 % of the plants under each treatment. The bars represent mean of 15 plants of each cultivar per sub-plot per treatment type and the error bars represent the standard error of mean.

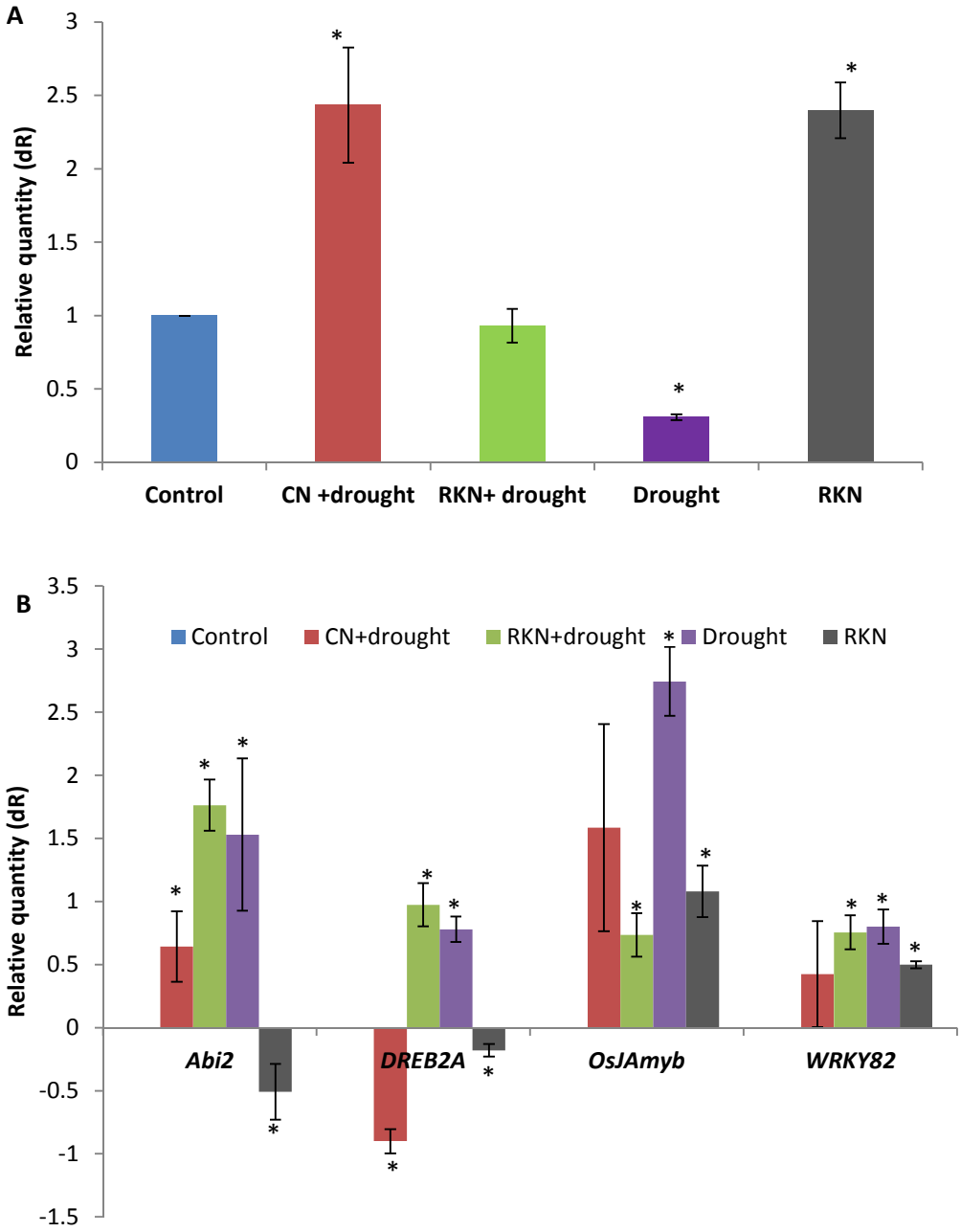


Figure 5-11. Quantitative expression analysis of stress responsive genes in hormonal signalling pathways in Nipponbare. A. Expression analysis of the *PR1b* gene, a marker of salicylic acid-mediated systemic acquired resistance in response to biotic stress**. B.** Expression analysis of ABA signalling-mediated abiotic stress response (*ABI2* and *DREB2A*), jasmonic acid signalling-mediated wounding response (*OsJAmyb*) and *WRKY82*, a marker for crosstalk between biotic and abiotic stress responses. Three technical replicates were performed on a pooled sample from three biological replicates.

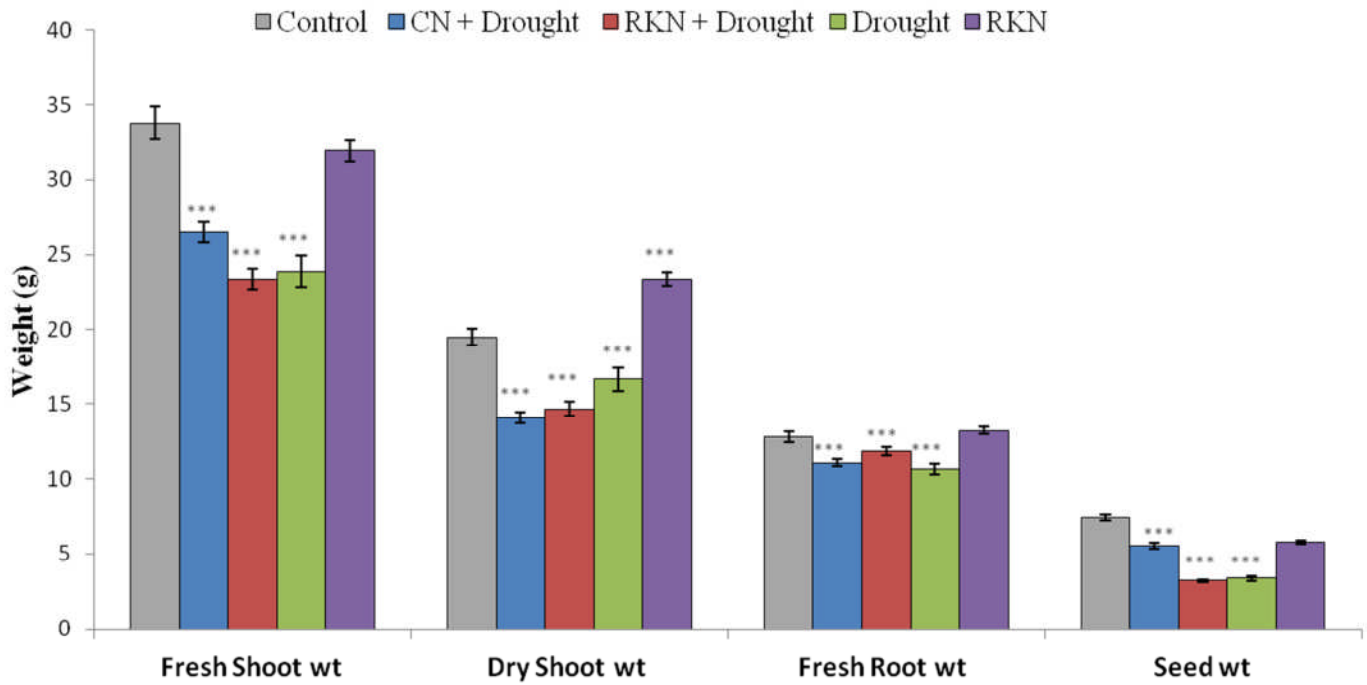


Figure 5-12. Effect of stress treatments on growth parameters of IR64 plants

The fresh and dry shoot weight, fresh root weight and seed weight per plant were recorded for nine plants in every sub-plot of IR64 in each treatment plot. Each bar represents the mean of 135 plants per treatment. Significant differences between control and treatment plants were analysed by Mann-Whitney U non-parametric test at (***P ≤ 0.005), error bards represent standard error of mean.

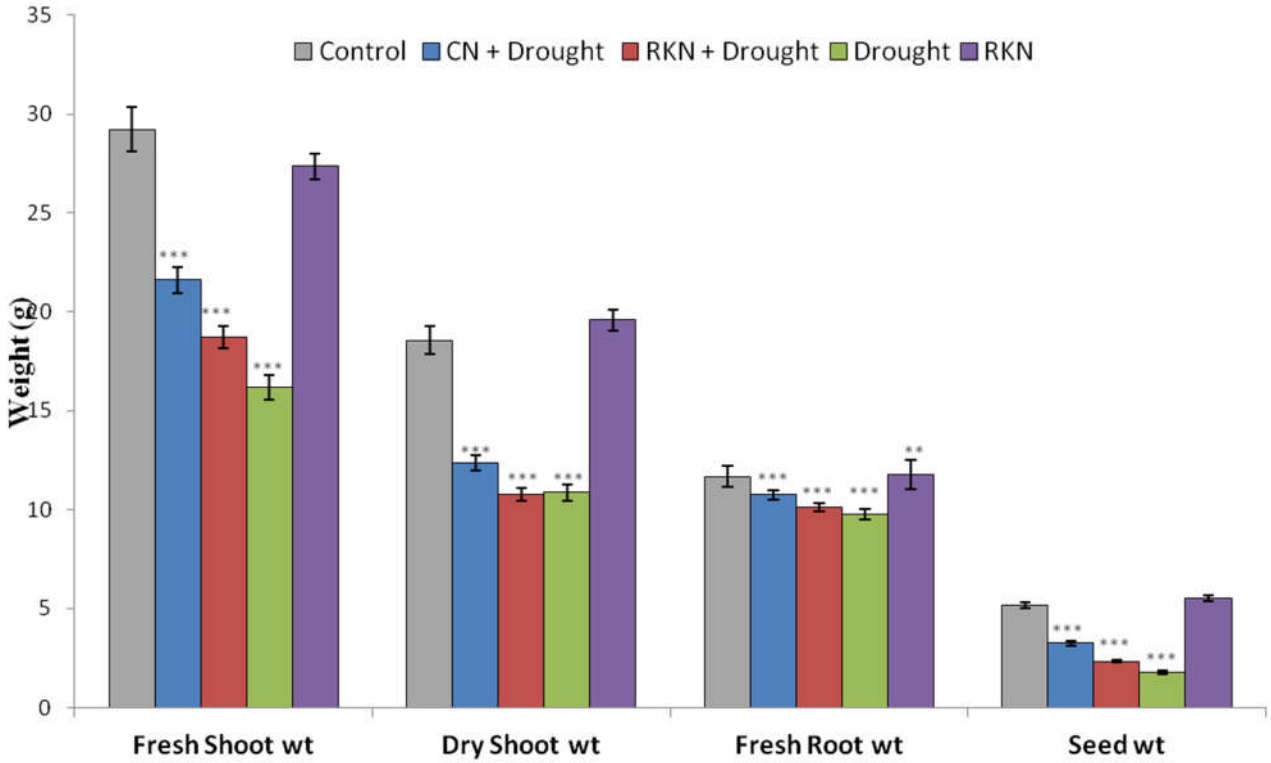


Figure 5-13. Effect of stress treatments on growth parameters of Nipponbare plants.

The fresh and dry shoot weight, fresh root weight and seed weight per plant were recorded for nine plants in every sub-plot of Nipponbare in each treatment plot. Each bar represents the mean of 135 plants per treatment. Significant differences between control and treatment plants were analysed by Mann-Whitney U non-parametric test at (***P ≤ 0.005), error bards represent standard error of mean. The significance was analysed by Mann- U Whitney non- parametric test at (** P≤ 0.01, ***P≤ 0.005), error bars represent standard error of mean. .

5.4. Discussion

Two lowland rice cultivars, IR64 and Nipponbare, were challenged in the field with nematode infection (CN and RKN) and vegetative drought stress, in combination and isolation. Nipponbare is a drought and nematode susceptible lowland japonica cultivar frequently used in laboratory and glasshouse studies to elucidate molecular responses in rice towards various biotic and abiotic stresses. It was grown alongside the drought and nematode susceptible Indica lowland cultivar IR64, used as the parent for traditional and molecular breeding programs (Fujita *et al.* 2010; Bimpong *et al.* 2010a). The growth, yield related change and hormonal signalling in response to simultaneous drought and nematode infection were investigated.

5.4.1. Molecular stress response

The molecular responses observed in Nipponbare plants in response to stresses in the field were similar to those elucidated by laboratory studies. The expression of the *PR1b* gene increased under all three treatments involving biotic stress, with significant induction in the plants under CND and the plants infected with RKN only. The plants that were only drought stressed showed decreased expression of the biotic stress marker gene *PR1b*, as observed in laboratory studies (Fujita *et al.* 2006). There was no significant change in expression of the *PR1b* gene in the plants under RKND stress. The initial density of RKN in the RKND plot was lower than that of cyst nematodes in CND and RKN in the only RKN infected plot, and taking into account the negative regulation of PR genes under abiotic stress, the response in the RKND treated plants could be explained as a result of additive effect of the biotic and abiotic stress on expression of PR genes. The expression of the abscisic acid mediated abiotic response marker; *ABI2* was increased in all drought stressed plants and reduced under only nematode infection (Lu *et al.* 2009). The expression of another drought responsive gene DREB2 was significantly reduced in CND infected plants and RKN infected plants but up-regulated RKND and drought infected plants. The JA pathway marker gene *OsJAmyb* is significantly increased in all nematode stressed treatments but highest expression as observed under drought stressed plants (Creelman and Mullet 1995). The results suggest that in simultaneous drought and nematode infection (low initial nematode density) the molecular responses are governed by the interaction of two stresses or the abiotic stress.

When plants are challenged with a cyst nematode infection (high initial nematode density) in combination with drought the stress responses are dominated by biotic stress responses.

5.4.2. Response of rice towards severe drought stress

Drought had a profound effect on the growth and yield of both rice cultivars. The plants under simultaneous drought and nematode infection (both CN + RKN) and under only drought stress showed reduced biomass and yield compared to the RKN infected and the control plants. The vegetative drought and reproductive drought stress are known to cause the highest yield losses in rice. Vegetative drought delays flowering and reduces biomass, whereas the reproductive drought reduces grain filling leading to reduced yield (Garrity and O'Toole 1995; Wopereis *et al.* 1996). A detailed pot and field study using different rice varieties has suggested that the drought sensitivity in different growth stages and the resulting yield loss is a varietal difference. The duration and severity of drought has a more severe effect on yield in all cultivars rather than the time of stress. Decreased water availability leads to reduced leaf area, reduced photosynthesis and leaf senescence, in turn resulting in decreased total biomass production and yield (Lilley and Fukai 1994) of Yield loss is observed in most cultivars at a soil saturation of 0-12 % and the yield decreases up to 40 % with further depletion of soil water (Bouman and Tuong 2001). In this study both cultivars; Nipponbare and IR64 were subjected to severe drought stress for the duration of 15 days followed by few showers and then intermediate drought for next 15 days. IR64 being a short duration variety (90 days) suffered a late vegetative and early reproductive stage severe drought whereas Nipponbare being a long duration variety (110 days) suffered a severe vegetative drought stress, but not a reproductive stage stress. The biomass can reduce up to 37 % under severe vegetative drought and up to 25 % in severe reproductive stresses in different rice varieties (Lilley and Fukai 1994). IR64 plants under severe vegetative and reproductive stress can suffer yield loss of 70 % and 90 %, respectively (Guan *et al.* 2010; Lilley and Fukai 1994). The severe vegetative drought stress causes higher damage in the Nipponbare plants, with 42 % reduction in the dry shoot mass and 65 % yield loss as compared to the unstressed control Nipponbare plants. Whereas, the drought stress of similar intensity occurring in late vegetative state of IR64 plants resulted in 25 % of dry shoot mass and 54 % reduction in the yield. Results indicate that

early vegetative stress has a more severe effect on yield loss in lowland rice than the late vegetative drought stress. In contrast to the results seen in other vegetative and reproductive drought stress studies (Hemmatollah Pirdashti 2009), no significant reduction was observed in the chlorophyll content of IR64 and Nipponbare plants. Although, reduction in the photosynthetic sufficiency, measured as the maximum quantum yield of photosystem II was seen over the duration of drought stress in both cultivars (Chen *et al.* 2011a; Hemmatollah Pirdashti 2009).

5.4.3. Response of rice to nematode infection

The feeding sites formed by plant parasitic nematodes act as nutrient sinks with the nematode mobilising photosynthate from shoot to root (Jones 1981). Reduced availability of nutrients for the plant reduces plant growth and yield. Rice seedlings inoculated with 3000 J2s of *M. graminicola* show stunting, yellowing and wilting with reduced root mass and shoot mass (Singh *et al.* 2006). Heavy *M. graminicola* infection (4687 J2/ plant) can reduce the shoot fresh, and dry weight and root weight by up to 70 % (Bimpong *et al.* 2010b). One-third reduction in root and shoot mass is observed under *H. sacchari* infection of rice seedlings in a pot experiment (Blouin *et al.* 2005). The dry shoot weight reduces up to 19 % in nematode susceptible cultivars under *H. sacchari* infection in fields. *H. sacchari* also shows increase in chlorophyll content of the stressed plants (Audebert *et al.* 2000). On contrary, the *M. graminicola* and *Helicotylenchus* are known to reduce the chlorophyll content in leaves of stressed plants (Dutta, Nayak and Prasad 1990). *H. sacchari* also reduces the maximum quantum yield of photo-system II *(Fm/Fv*) to 0.77 from 0.88 in the control uninfected plants (Blouin *et al.* 2005). In this study no reduction in the biomass of plant infected with RKN, *M. incognita* was seen. Instead, the dry shoot weight in IR64 plants and fresh root weight in Nipponbare plants was significantly higher under nematode infection as compared to the corresponding uninfected controls. The RKN infection did not have any influence on the chlorophyll content of the plants but the maximum quantum yield of the IR64 plants was significantly reduced with the duration of RKN infection. The damage caused by nematodes is dependent on the growth stage at which the nematodes infect plant and the density of initial inoculum (Singh *et al.* 2006; Jaiswal, Kumar and Singh 2012). The nematode density in only nematode treatment plot was moderate and the nematode population in this plot was dominated by the *M. incognita*, which is a less

virulent pest of rice than compared to the *M. graminicola.* Low populations of *Heterodera* and *Meloidogyne* species have shown stimulation of plant growth instead of detrimental effect (Barker and Olthof 1976).

5.4.4. Response of rice to simultaneous drought and nematode infection

When acting simultaneously, nematode infection can exaggerate or counteract the damage caused by drought stress through disturbance of the plant-water relations (Smit and Vamerali 1998; Atkinson and Urwin 2012). The cyst nematode *Globodera pallida* can lead to retarded growth but increased longevity of potato roots under simultaneous drought stress. Using individual root segments it was determined that presence of nematode delayed potato root decay. (Smit and Vamerali 1998). A study conducted on *H. sacchari* susceptible *Oryza sativa cultivar* and resistant *Oryza glaberrima* under drought and nematode stress showed that the cyst nematode when in combination with drought intensified the physiological responses to drought with reduced leaf water potential, osmotic potential and partial stomata closure. Under simultaneous stress, the chlorophyll content also decreased in the susceptible *Oryza sativa* cultivar and increased in the tolerant *Oryza glaberrima* cultivar as compared to the individual stress treatments. The leaf and root dry weight was reduced in both cultivars under simultaneous stress. Simultaneous drought and CN infection caused a higher yield loss than each of the stresses individually, indicating an additive effect of the two stresses on the plant (Audebert *et al.* 2000). The virulence of *M. graminicola* on rice is dependent on water availability and the damage caused by the nematode intensifies in dry, aerobic soil (Soriano, Prot and Matias 2000). A study on 15 rice cultivars infected with *M. incognita*, identified a pattern of increased fresh shoot and root weight when plants were infected with 5000 J2s (R.A. Adebayo 2010). In this study, higher yield loss was observed in the simultaneous drought and RKN infected plants (IR64 57 % and Nipponbare 54 %) than the simultaneous drought and CN infection (IR64 37 % and Nipponbare 25 %). The growth parameters in both groups of simultaneous stressed plants were reduced compared to control plants, but the RKND treatment had a more severe effect on plant growth than the CND treatment. Chlorophyll content of leaves was reduced in both simultaneous stress treatments with the CND treatment showing a more significant reduction than the RKND treatment. The simultaneous drought and

nematode stress severely reduces nitrogen availability to the plant indicating reduced absorption of nutrients from the soil. These results are in accordance with the data obtained in a study of *Oryza sativa* cv. Monobereken under *H. sacchari* infection but are contrary to a similar study done with *H. sacchari* and drought (Audebert *et al.* 2000; Blouin *et al.* 2005). The maximum quantum yield of the IR64 plants under simultaneous CN and drought stress was reduced to nearly half of the uninfected IR64 plants, whereas the Nipponbare plant under same treatment did not show a significant reduction. The maximum quantum yield (*Fv/Fm*) of photosystem II in healthy plants is 0.80 (Maxwell and Johnson 2000). Under the RKND treatment the maximum quantum yield of both cultivars were equally affected. The CN infection had a more severe effect on the photosynthetic properties of both cultivars but it improved the plants' response towards drought and resulted in less yield loss and higher biomass gain compared to the control and simultaneous RKN and drought infected plants. The moderate RKN infection on rice plants in combination with the drought stress is a more severe stress on plants than the simultaneous drought and cyst nematode infection.

H. sacchari is a major nematode pest of upland rice and *M. incognita* is prevalent in rice cultivation in hydromorphic soils in West Africa (Coyne, Smith and Plowright 2001). There are no records available for *H. sacchari* and *M. incognita* infestation on IR64 or Nipponbare cultivars as both are predominantly cultivated in Asia. In the South Asian sub-continent, *M. graminicola* is the major pest that affects rice under aerobic and upland conditions and leads to maximum yield loss. Due to the absence of a cyst nematode infected plot that did not receive a drought stress, it is difficult to conclude that the differences seen in the growth and yield parameters under the two simultaneous stresses is due to the presence of two different types of nematodes. The nematode-plant interaction models suggest that plants respond differently to the PPN density. In some plant species shoot weight increased with increase in nematode density, whereas in others root weight increases with increase in nematode density and in others the nematode density has a negative influence on the plant growth (Wallace 1971). The nematode density in the CND plots was higher than the RKND infected plots. The CND treatment plot had 343 cysts / 100 g of soil, *H. sacchari* cysts can have 91-222 eggs/ juveniles per cyst depending on the size of the cyst (Ibrahim *et al.* 1993). This difference in density also makes it difficult to conclude the responses seen are due to the nematode type or the nematode density.

6 Chapter

Reduction of phytate by down-regulation of *Arabidopsis thaliana MIPS* and *IPK1* genes alters susceptibility to cyst nematodes

6.1. Introduction

According to the Food and Agriculture Organisation (FAO), 33 countries in the world are presently facing food crisis (Bruulsema *et al.* 2012). Globally, 870 million people i.e. one in every eight individuals, are under-nourished (FAO 2012). The pressure on the world's agricultural community is increasing with growing population, reduced agricultural land and global climate changes. To assure food security across the globe the food grain production has to increase along with the nutritional value of the food grains. Deficiency of micronutrients like iron, zinc, iodine, copper and magnesium is the main cause of malnutrition in humans, especially in developing nations (Bhutta, Salam and Das 2013). Apart from low content of these micro-nutrients in plant and animal sources, their bioavailability in food also decreases due to processing and cooking, and due to reaction with other components in the food or by the presence of anti-nutrients in the diet that limits absorption of these essential micronutrients (Bruulsema *et al.* 2012). One such anti-nutrient is phytate or phytic acid. Phytate has a profound influence on animal health and the environment. Excess dietary intake of phytate in humans results in mineral deficiency and formation of insoluble non-specific phytate complexes which are not readily hydrolysed and reduces absorption of proteins, carbohydrates and lipids associated with them (Kumar *et al.* 2010). Non-ruminant animals are unable to metabolize phytate and non-phytate phosphorous present in plants is not sufficient to fulfil the phosphorous requirement for optimal animal productivity. This undigested phytate is secreted and use of animal manure in agricultural systems allows it to run off into water bodies where it is broken down by algae and causes deoxygenation of aquatic ecosystems (Leytem and Maguire 2007). From a nutritional and environmental point of view there has been an urge to produce crops with reduced levels of phytate in both seeds and vegetative tissue.

Phytate, also known as inositol hexakisphosphate (IP_6), is a phosphorylated derivative of *myo*-inositol. It accounts for 75 % of the total phosphorus stored in seeds (Raboy 2009). Phosphorus taken up by plants is stored in developing seeds in the form of phytate. Different phosphorylated derivatives of inositol in seeds form a major pool of phosphorous flux through the world's agricultural ecology. In mature seeds phytate is stored as mixed salts of K, Mg, Ca, Fe, Mn and Zn in discrete inclusions called globoids within protein storage vacuoles, in a tissue-specific manner (Raboy 2003). During germination phytate breaks down to maintain constant levels of inorganic phosphorous and cellular phosphorous. Phytate is ubiquitously present in all eukaryotic species where it is involved in various developmental and signalling processes including directional translocation of nuclear RNA, inhibition of phosphatase activity in a concentration dependent manner and increase in calcium channel activity initiating other signalling pathways and RNA editing (Macbeth *et al.* 2005; Alcázar-Román *et al.* 2006; Larsson *et al.* 1997). Phytate levels in guard cells of plants elevate in response to ABA which leads to closing of stomata during environmental stress (Lemtiri-Chlieh, MacRobbie and Brearley 2000; Lemtiri-Chlieh *et al.* 2003). Phytate is also involved with auxin-mediated regulation as a co-factor in TIR1 auxin receptors (Tan *et al.* 2007).

Phytate is synthesized in cells by two pathways, lipid-dependent and lipid-independent. In a highly conserved two-step biochemical pathway, known as the Loewus pathway, glucose-6-phosphate is converted to *myo*-inositol-3-phosphate. This is a rate limiting reaction catalysed by D-*myo*-inositol-3-phosphate synthase (MIPS). Myo-inositol undergoes a series of epimerisation reactions and phosphorylation of the hydroxide moieties to form 37 distinct, important cellular metabolites including phytate (Valluru and Van den Ende 2011). The *myo*-inositol-3-phosphate acts as a substrate for the lipid-dependent phosphatidylinositol phosphate pathway and lipid-independent inositol phosphate pathway. These two pathways lead to formation of a number of phosphorylated inositol derivatives including inositol pentakisphosphate. Conversion of inositol pentakisphosphate to inositol hexakisphosphate is the final step of phosphorylation in phytate biosynthesis catalysed by inositol polyphosphatasekinase (IPK_5, 2-kinase). The phytate produced can be further phosphorylated to form higher phosphorylated derivatives called pyrophosphate-containing inositols (Raboy 2003) (Figure 6.10).

Various steps of phytate biosynthesis can be targeted in order to generate low phytate plants. Favourable strategies include targeting the Loewus pathway or the conversion of inositol and inositol phosphate to phytate, manipulation of transport of the produced phytate and introduction of microbial and plant phytase (Raboy 2009). The Loewus pathway is the only source of the inositol ring in plants and manipulation within it to reduce total phytate content can be deleterious if not lethal. RNAi-mediated silencing of a *myo*-inositol-phosphate synthase gene (*GmMIPS*) in soybean leads to reduced phytate content but also inhibits normal seed development (Nunes *et al.* 2006). In contrast, transgenic low phytate rice, with 68 % reduced phytate generated using antisense suppression of the 1D-*myo*-inositol-3-phosphate synthase gene (*RINO1*) had no negative effect on seed weight, germination and plant growth (Kuwano *et al.* 2009a). Mutation in the inositol phosphate kinase gene to manipulate the amount of inositol pentakisphosphate converted to inositol hexakisphosphate has been exploited in various crop species. Low phytic acid mutants have been identified in maize, rice, soybean, wheat, barley, *Arabidopsis thaliana*, common bean (*Phaseolus vulgaris*) and very recently in pea using chemical mutagenesis (Murphy *et al.* 2008; Raboy 2009; Warkentin *et al.* 2012). Maize *low phytic acid* (*lpa*) genotypes with mutations in polyphosphate kinase genes, *ipa1-1*, *ipa1-2* have 50 to 66 % reduced seed phytate and show normal seed development but have delayed flowering, lower stress tolerance and reduced yield (Raboy *et al.* 2000). Transgenic wheat, soya bean, maize and Arabidopsis have been developed by introducing microbial phytase to reduce phytate accumulation in seeds and increase phosphate availability in animals (Coello *et al.* 2001; Chen *et al.* 2008b; Denbow *et al.* 1998; Brinch-Pedersen *et al.* 2003; Drakakaki *et al.* 2005).

Arabidopsis has three copies of the *MIPS* gene; *MIPS1* was identified by genetic complementation of the yeast *INO1* gene (Valluru and Van den Ende 2011). The function of the MIPS enzyme is conserved in yeast, animals, fungi and plants (Loewus and Loewus 1983; Michell 2008). Plants can have multiple *MIPS* gene copies whereas yeast and animals have just one. The three *MIPS* gene copies in Arabidopsis result in the expression of three enzyme isoforms with 90 % amino acid similarity. They have non-redundant function and they contribute to different cellular pools of phytate (Mitsuhashi *et al.* 2008). Low phytate Arabidopsis mutants (*mips1*, *mips2-1*, *mips2-2 and mips3*) have been developed by knocking out *MIPS* genes. Disruption of *MIPS1* leads to embryonic developmental defects and suppression of programmed cell death in

later developmental stages (Luo *et al.* 2011; Donahue *et al.* 2010). Overall reduction of phytate levels in cereals have resulted in compromised yield due to reduced stress tolerance (Raboy, Ertl and Young 1998; Raboy and Bregitzer 2006; Murphy *et al.* 2008). Low phytate potatoes produced by either introducing an *anti-IPS* transgene or expressing a bacterial polyphosphate kinase *PPK* gene show enhanced susceptibility to viral pathogens (Murphy *et al.* 2008). The low phytate Arabidopsis *MIPS* mutants also show enhanced susceptibility to viral, bacterial and fungal pathogens infecting the aerial parts of the plant. Although *mips1* and *mips2* mutants have similarly reduced levels of plant phytate they show different responses to microbial pathogens. In one study, the *mips1* mutant was no more susceptible to various pathogens than wild type plants whereas the *mips2* mutant was highly susceptible to RNA and DNA viruses, bacterial and fungal pathogens (Murphy *et al.* 2008). However, in a different study plants mutant for the *MIPS1* gene showed reduced innate immunity when infected with the virulent fungal pathogen *Hyaloperonospora parasitica* (Meng *et al.* 2009). The *mips2-1* and *mips2-2* mutants are both knock-outs for *MIPS2*; only *mips2-1* displays increased expression of *MIPS1* in comparison to the levels in a wild type plant.

In Arabidopsis, the *IPK1* and *IPK2β* genes encode inositol pentakisphosphate-2-kinase enzymes that catalyse the last phosphorylation reaction in phytate synthesis, directly leading to production of inositol hexakisphosphate (Stevenson-Paulik et al. 2005). Low phytate Arabidopsis mutants have been developed by knocking out the *IPK1* gene. *ipk1* mutant seedlings have 93 % reduction in tissue phytate level compared to wild type. The absence of *IPK2β* reduces seed phytate level to 35 %, however its function is compensated in seedlings and consequently they have normal levels of phytate (Stevenson-Paulik *et al.* 2005). The *ipk1* mutant shows increased susceptibility to virulent and avirulent bacterial strains and fungal pathogens (Murphy *et al.* 2008). No data is available for the response of other low phytate mutants (*mips2-2* and *mips3*) towards microbial pathogens, similarly not much is known about the effects of reduced phytate levels on root parasites. In this study we have investigated the effect of low phytate content on nematode infection in plants.

We describe the infection of loss of function Arabidopsis mutants disrupted in genes involved in the first and last step of phytate synthesis with the cyst nematode *Heterodera schachtii* and root-knot-nematode *Meloidogyne* species. We have compared the results to those obtained with a salicylic acid transgenic *NahG* that is unable to

accumulate salicylic acid and shows higher susceptibility to fungal, bacterial and viral pathogens, as well as cyst nematodes.

6.2. Materials and methods

6.2.1. Plant material and growth conditions

The *mips1-2* (SALK_023626), *mips 2-1* (SALK_031685*), mips2-2* (SALK_108779) and *mips3* (SALK_120131) mutants were obtained from Nottingham Arabidopsis Stock Centre (NASC). *ipk1* and *NahG* seeds were gifts from John P Carr, University of Cambridge. Seeds were surface sterilised by incubation in 20 % commercial bleach for 20 mins followed by five washes in sterile deionised water. Seeds were stratified at 4 °C and subsequently grown on square Petri dishes on solidified half strength Murashige and Skoog medium with 1 % sucrose in an upright position (Figure 6-1 A). Growth took place in a Sanyo controlled growth chamber at 20 °C under 16 hour/8 hour light/dark cycles at an average light intensity of 140 µmol/m^2/s at 30 % humidity. In soil experiments plants were grown in similar conditions in a Sanyo growth chamber (Figure 6-1 B). Two seeds of the same type were grown on each tissue culture plate and in the soil experiment one seedling was grown in each 9 cm pot containing potting compost.

6.2.2. Nematode collection

Heterodera schachtii cysts were extracted from soil, sterilised and hatched as described in Urwin et al (1997). *M. graminicola* stocks were maintained on *Oryza sativa* (rice). The second stage juveniles were hatched from infected roots using a mistifier technique (Luc, Sikora and Bridge 2005) After hatching, juveniles were sterilised in 0.1 % chlorhexidine digluconate and 0.5 mg/ml hexadecyltrimethylammonium bromide (CTAB) for 30 min on a rotor mixer at room temperature. The juveniles were then washed five times in filter sterilised tap water with 0.01 % Tween-20 and re-suspended to a concentration of 1 nematode/µl.

6.2.3. Nematode assay

At Boyes growth stage 1.04, Arabidopsis plants (25 plants per mutant per replicate)) grown in tissue culture were infected with 25 nematodes at each of five infection points (root tips) per plant. A small square piece of GF/A filter paper (Whatman) was placed on each inflection point to aid nematode penetration, and was removed after 48 hours.

Nematodes were allowed to develop on the roots for 14 days after which each root system was weighed and stained using a modified acid fuschin staining method (Section 2.2.6). The Arabidopsis roots were soaked in 1 % sodium hypo-chlorite solution for only 3 min, instead of 5 min and rinsed. The stained roots were observed under a stereobinocular Leica MZ16 microscope and the nematodes (Figure 6-1. C-F) that had developed on each root system were counted and assigned to developmental stages. Four replicates were performed with each nematode species.

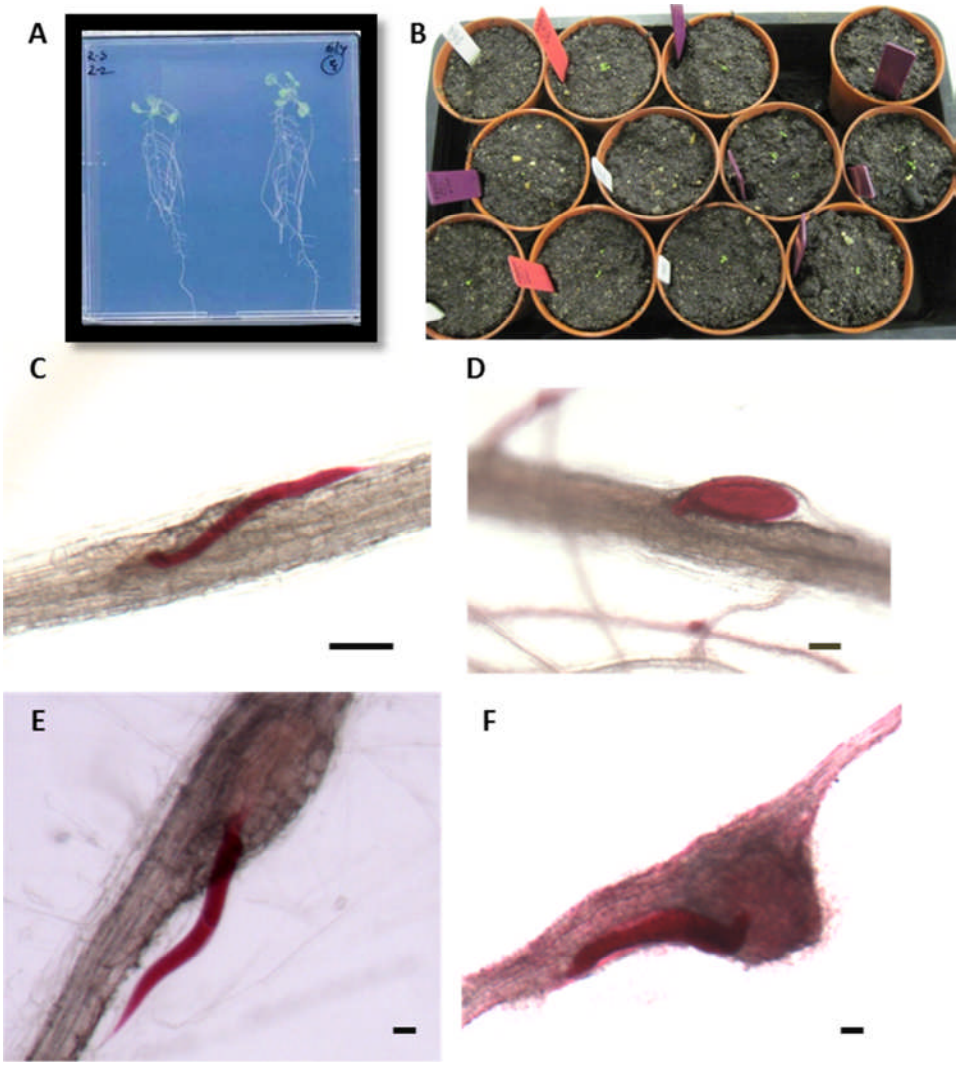


Figure 6-1. Set up for growth study of low phytate mutants and nematode infection study. A. Plate-based early growth analysis system – two seedlings were grown on half MS media, the growth stages were monitored after sowing. At growth stage 1.04 the plates were scanned and each root system was measured. For infection studies, each plant was infected with 100 nematodes. **B**. For late growth analysis plants were grown in compost in 3 inch pots, arranged at random. For the infection study, the number of nematodes at various growth stages was counted in stained roots. **C.** *H. schachtii* second stage juvenile. **D.** *H. schachtii* fourth stage juvenile. **E.** *M. graminicola* second stage juvenile initiating a gall **F.** *M. graminicola* third stage juvenile in a gall. The scale bars denote 100 µm.

6.2.4. Genotyping of the *mips1-2* mutant

Genomic DNA was extracted from leaves of mutant plants using the method specified by Meng *et al*. (2009). The *mips1-2* mutants were screened for homozygosity of T-DNA insertion using T-DNA right border primer (LBb1-GCGTGGACCGCTTGCTGCAACT) and gene specific primers (TTGCTAGCAACCATATCGTC and TTCGTGTCGGATCTTTTAACG). The PCR was conducted by 2 minute activation at 95 ºC followed by 35 cycles of 95 ºC for 1 minute, 52 ºC for 1minute 30 seconds, 72 ºC for 1 minute and a final step of 72 ºC for 5 minutes, using a GoTaq Hot Start polymerase master mix (Promega). PCR products were analysed by agarose gel electrophoresis (Section 2.2.3.2).

6.2.5. Growth analyses of mutants

For analysis of germination and early root growth, the mutant plants along with wild-type Col-0 Arabidopsis were grown on ½ MS10 medium as described earlier and growth stages were recorded until the growth stage 1.04 was reached i.e. more than 50% of wild type seedlings had primary roots ≥ 6 cm in length. Twenty plants of each genotype were scanned on the culture plates and roots were measured using Image Pro Analyser 7.0 software. For comparison of late growth stages, plants were grown in soil as described (Twenty plants each genotype). Growth stages were recorded after emergence of first cotyledon (growth stage 1.02) until 14 rosette leaves (growth stage 1.14) were visible (Boyes *et al.* 2001). After 60 days height of primary inflorescence and mean number of seeds was calculated from ten ripe siliques collected per genotype . The experiment was repeated twice.

6.2.6. Statistical analyses

The data were analysed using IBM statistic package SPSS18. All the data obtained were not normally distributed thus the non-parametric Kruskall-Wallis and Mann-Whitney U tests were used to determine significant differences ($p \leq 0.05$).

6.3. Results

6.3.1. Effect of low phytate content on growth and development of Arabidopsis mutants

Growth analysis was performed on low phytate Arabidopsis mutants to determine the effect of compromised phytate levels on plant growth. A salicylic-acid deficient transgenic line expressing the bacterial *NahG* gene was included for comparison. The principle Boyes growth stages of the mutants were analysed in plate-based and soil-based experiments and compared with the wild type Col-0 plants. All four genotypes (*ipk1, mips1, mips2-1*and *NahG*) showed developmental differences from wild type plants (Figure 6-2A-2B). Both *ipk1* mutants and *NahG* transgenic plants developed more slowly than the wild type in the early growth stages on a plate-based system. *NahG* transgenic plants took 11.1 days to reach growth stage 1.04 whereas wild type took an average of 9.25 days which is significantly less ($P \leq 0.05$) and *ipk1* mutant took 11.4 days. In the soil-based system the first rosette leaves in *mips1* plants took thirteen days to develop in comparison to ten days for wild type plants. After emergence of the first rosette leaves took place, the development continued at a comparable rate to wild type. The Boyes growth stage 1.04 is marked by 4 rosette leaves ≥ 1mm, *ipk1* (16.58 ± 0.82 days) and *NahG* (19.33 ± 0.66 days) plants were significantly quicker and slower respectively to reach this stage when compared to wild type (18.88 ± 0.53 days), but thereafter the development continued at a similar rate to the wildtype. The root system of each mutant and wildtype plant was analysed at growth stage 1.04 by counting the number of lateral roots and measuring the length of the primary root (Figure 6-3A-B). *NahG* (10.92 ± 0.58) and *mips2* (8.15 ± 1.01) plants had bigger root systems with more lateral roots than the wildtype (6.92 ± 1.2). The *mips1* plants had significantly fewer lateral roots (3.46 ± 0.45; $P \leq 0.005$). In the soil-based experiment, the height of the primary inflorescence and number of seeds per silique were recorded after 60 days of growth period. In comparison to wild type plants, mutant plants and the *NahG* transgenic line showed no difference in the height of primary inflorescence and number of seeds per silique. (Figure 6-3C-D).

In tissue culture and soil-based growth conditions the *mips1* mutant plants showed two different phenotypes, 25 % of total number of plants were smaller in size, had abnormal cotyledon boundaries and showed lesions (Figure 6-4D-F). For this reason, the

homozygosity of T-DNA insertion in mutant *mips1* was tested by performing PCR using a gene-specific primer and a T-DNA insert-specific primer. All plants were homozygous for the T-DNA insert. The wildtype plants and *mips2-1* mutants showed a single band ~ 1100bp, amplified by *MIPS1* gene specific left border and right border primer pair. No amplification was seen in the DNA obtained from *mips1* mutants with normal as well as abnormal phenotype, using the gene specific primer pairs (Figure 6-4 B). Using the gene specific left border primer and T-DNA right border primer, a single band ~600 bp was amplified in the *mips1* mutant with normal and abnormal phenotype/ Whereas, no amplification was seen in the wildtype and *mips2-1* plants uding this primer pair (Figure 6-4 C) A similar phenotype has been observed in homozygous *mips1* mutants by other groups when grown in high light intensity (Donahue *et al.* 2010). This phenotype was seen in earlier growth stages, in later stages of development *mips1* mutants were undistinguishable from Col-0 in phenotype. In the soil-based growth analysis set-up, the *mips1* cotyledons also showed lesions (Figure 6-3G).

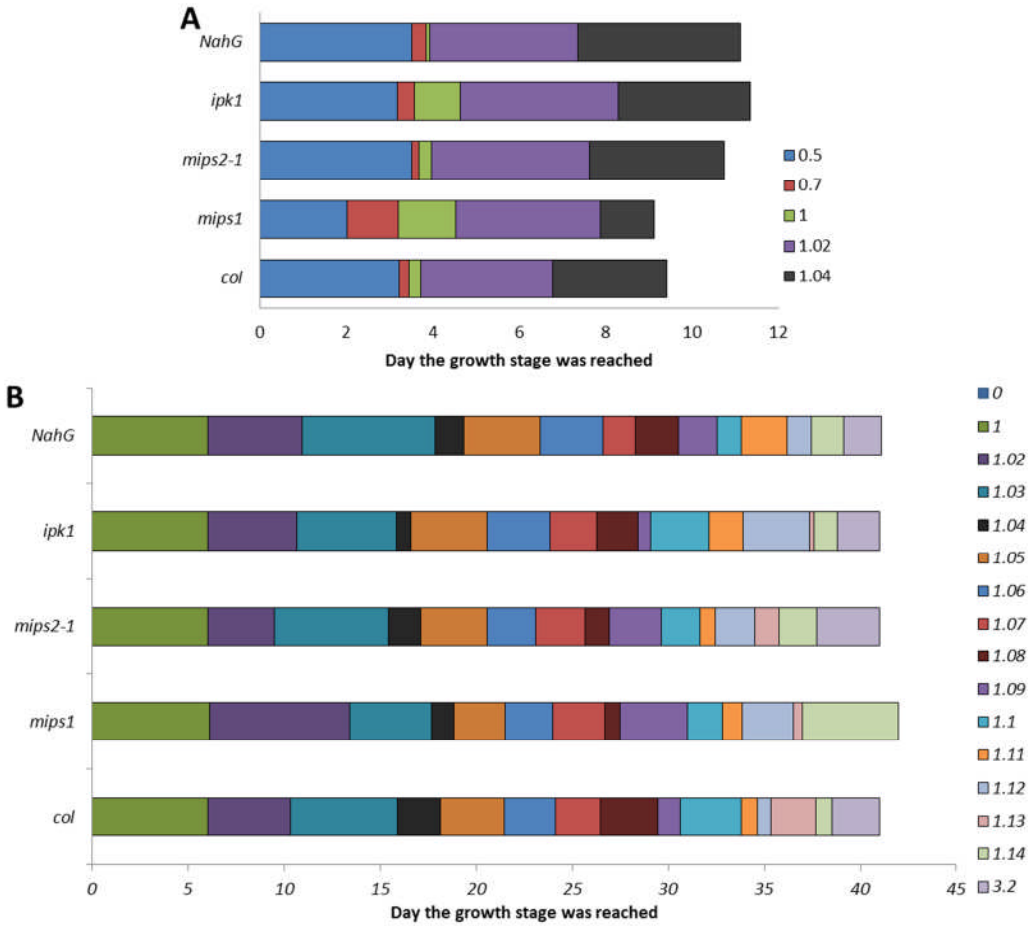


Figure 6-2. Growth stage progression for wild type (*col*) and low phytate mutants

A. Growth stage progression as determined on plate-based early growth analysis system. **B.** Growth stage progression as determined in the soil-based later growth analysis system. The boxes of different colour indicate time elapsed between the successive Boyes growth stages (Boyes, 2001).

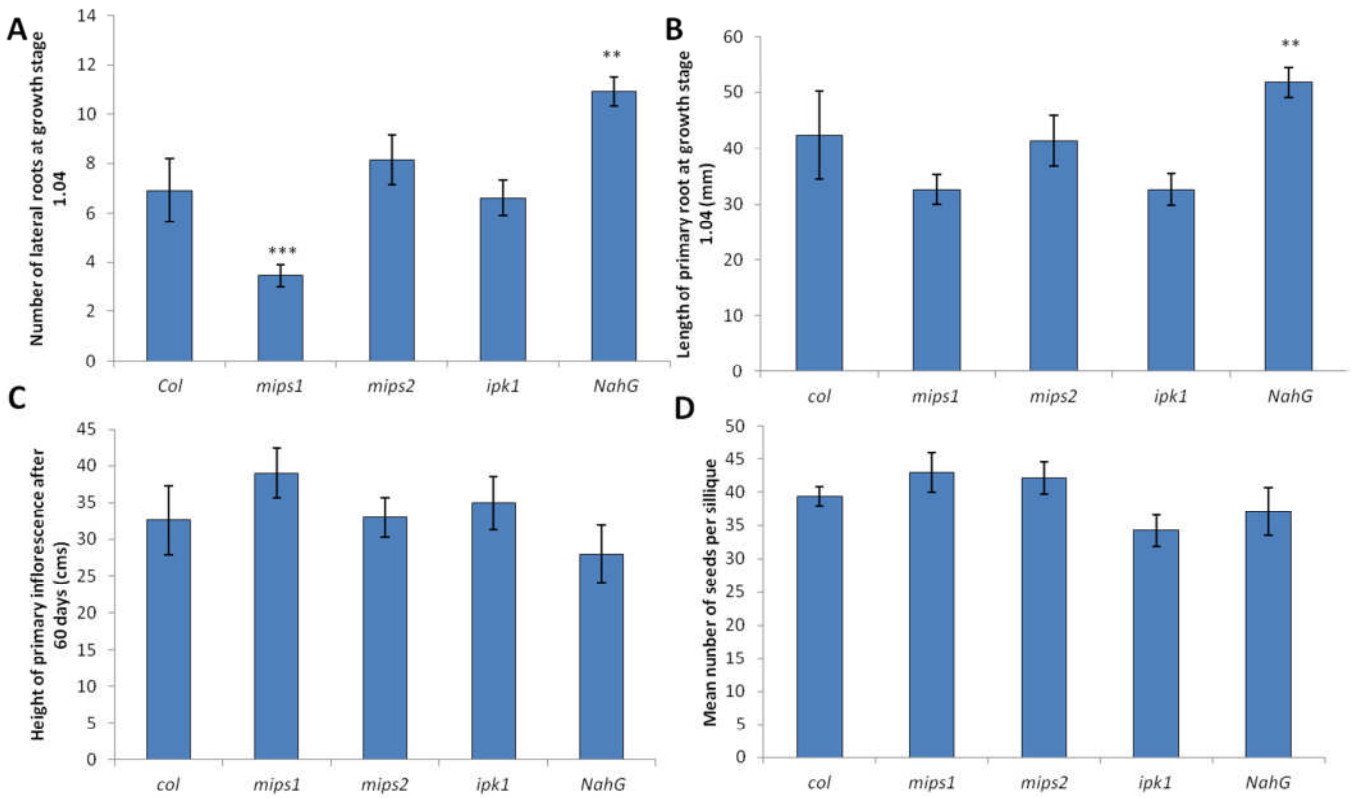


Figure 6-3. Growth analyses of low phytate mutant genotypes in comparison with wild type col and salicylic acid transgenic NahG plants.

- **A.** Mean number of lateral roots in mutant and wild type plants at Bayes growth stage 1.04. (n=24)
- **B.** .Mean length of primary roots in mutant and wild type genotypes at Bayes growth stage 1.04. (n=12)
- **C.** .Mean height of primary inflorescence in mutant and wild type genotype plants at 60 days post germination. (n=30)
- **D.** Mean number of seeds per silique. (n=30, 10 siliques per plant). each genotype was compared to wild type using Mann-Whitney U test. (** = $p \leq 0.01$, *** = $p \leq 0.005$).

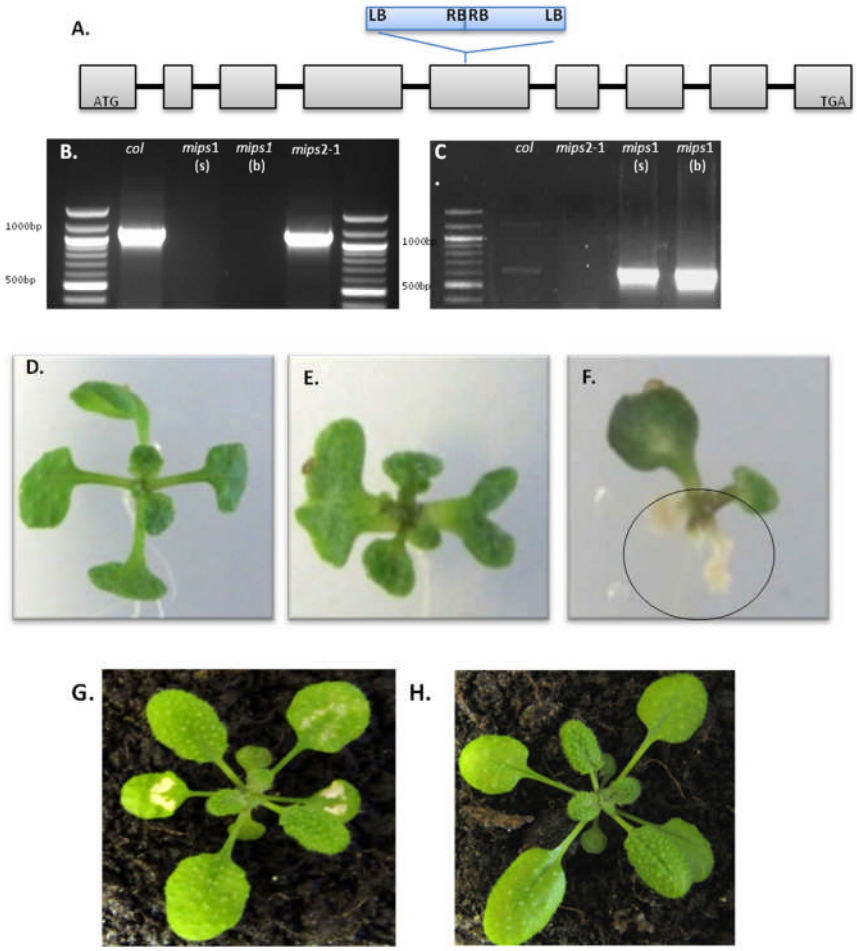


Figure 6-4.Molecular characterisation and seedling phenotype of wild type Col-0 and *mips1* mutant plants.

A. Structure of the *MIPS1* gene in *Arabidopsis thaliana*. The boxes represent exons; the T-DNA insertion site is marked with orientation of the insert. Homozygosity of T-DNA insertion. B. Gel picture showing products amplified from Col-0, *mips1* (normal (b) and abnormal (s) phenotype) and *mips2* mutants using gene specific primers for wild-type *MIPS1 locus*. C. Gel picture showing products amplified from Col-0, *mips1* (normal and abnormal phenotype) and *mips2* mutants using right border T-DNA insert primer and *MIPS1* forward gene-specific primer. D. Cotyledon development of wild type seedling. E. Abnormal cotyledon margin of *mips1* mutant. F. Small cotyledon of *mips1* mutant showing lesion in encircled area. G. Cotyledon lesions on *mips1* in soil-based growth analysis. H. Normal cotyledon in wild type Arabidopsis plants under soil-based growth analysis system.

6.3.2. Effect of disruption of the first and last steps of phytate synthesis on *H. schachtii* parasitism

To study the effect of low phytate content on nematode infection, low phytate Arabidopsis mutants were infected with *H. schachtii*. The nematode burdens of low phytate mutants (*mips1, mips2-1, mips2-2, mips3* and *ipk1*), the salicylic acid deficient *NahG* transgenic line and a wild-type control were determined 14 days after inoculation with *H. schachtii* second stage juveniles. The total number of nematodes entering and developing within the roots was taken as a measure of susceptibility. The number of nematodes that had achieved different life stages was also determined. The salicylic acid deficient transgenic *NahG* line, which is hyper-susceptible to *H. schachtii* infection (Wubben, Jin and Baum 2008), was also incorporated into the study as a positive control.

The total numbers of *H. schachtii* in different developmental stages were counted in stained roots in each mutant genotype (Figure 6-5). The *mips2-1* mutant roots had significantly fewer J2 (2.78 ± 0.54), J3 (2.04 ± 0.42) and J4 female nematodes (0.073 ± 0.04) than the wild type plants (J2, 5.27 ± 0.50; J3, 4.04 ± 0.52 and J4 females, (0.5 ± 0.14). The *ipk1* mutant plants had significantly more J3 (7.06 ± 0.71) and J4 female nematodes (0.86 ± 0.18) than the wild type plants. The positive control transgenic *NahG* plants had more J2 (11.9 ± 1.71), J3 (9.48 ± 1.17) and J4 females (0.73 ± 0.19) than the wild type plants. The total number of nematodes and the proportion of different life stages on *mips1, mips2-2* and *mips3* were similar to WT and were significantly different from *NahG* (statistics not shown). In comparison to WT (9.7 ± 0.81), *mips2-1* mutants supported lower infection, with a reduced total number of nematodes (4.9 ± 0.89) and no J4 female nematodes observed on the plants. The absence of *MIPS2* therefore led to a reduction in the number of nematodes observed in the roots and slowed the rate of development. Increased infection was observed in the *ipk1* mutant as compared with the WT; the infection levels were similar to the hyper-susceptible *NahG* transgenic line (Figure 6-6 A). The *mips3* plants had a higher proportion of second stage juveniles (65.21 ± 6.5 %) in the total population compared to the wild type (54.06 ± 3.68 %). Later developmental stages of *H. schachtii* were studied after 28 days of infection on mutant plants. The *mips2-2* mutant (3.34 ± 0.47) and *NahG* transgenic line (5.62 ± 0.75) each had a higher number of adult female nematodes per plant than the wild type (1.07 ± 0.22) (Figure 5.7A). The size of adult females

developing on *ipk1* mutant plant roots was reduced by 50 % in comparison to the females found on the wild type plants (*ipk1*, 92,309.34 ± 25477 mm^2; wild type, 197,844.3 ± 22193.72 mm^2)(Figure 6.7B & 6.8).

6.3.3. Effect of disruption of the first and last steps of phytate synthesis on *Meloidogyne graminicola* parasitism

Given the observed pattern of increased susceptibility to all type of pathogens in *ipk1* mutants, we investigated the response of other low phytate mutants towards root-knot-nematode infection. All mutant genotypes were infected with *M. graminicola*, the numbers of galls and total number of nematodes in each root system was determined at 14 dpi. There was no significant difference in total nematodes on any of the mutant genotypes in comparison to the wild type Arabidopsis plants ($p \leq 0.05$) (Figure 6-9A). The data was analysed to determine the proportion of the different developmental stages of nematodes; the proportion of second stage juveniles and third stage juveniles on the mutant genotypes was not significantly different from the proportion on the wild type plants (Figure 6-9B). There was no significant difference in the total number of galls per plant on each mutant (Figure 6-9C). Thus, the low phytate plants were no more resistant or susceptible to infection by the root-knot nematode species than wild type Arabidopsis.

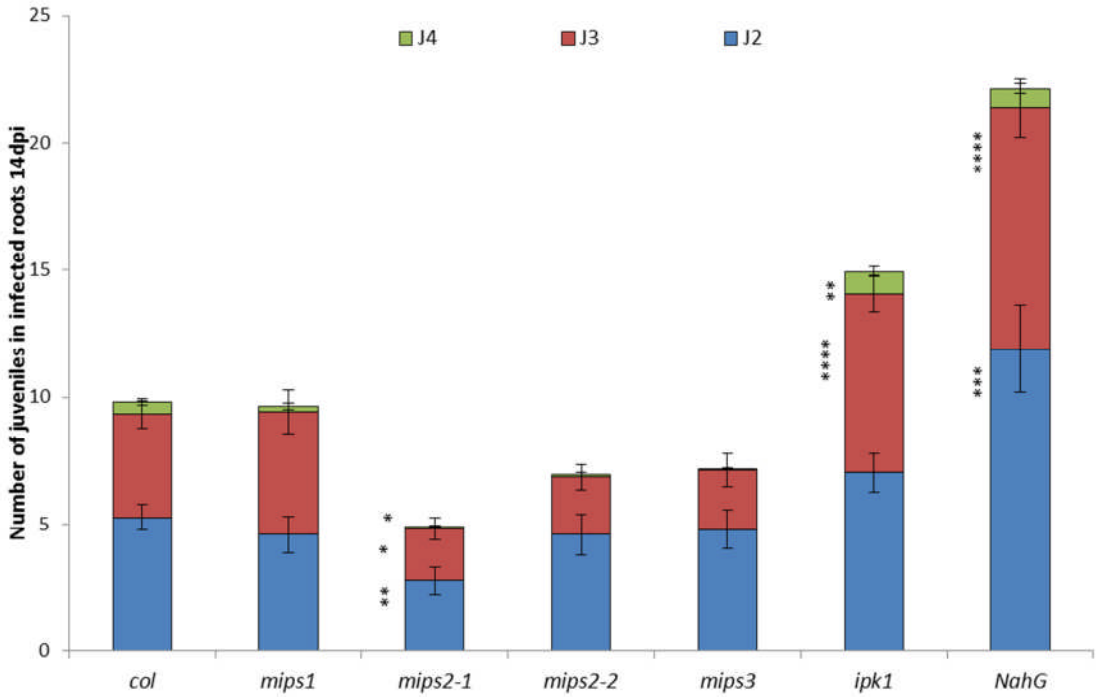


Figure 6-5. Rate of infection and development of H. schachtii. on low phytate mutant Arabidopsis compared to wild type Col-0 and salicylic acid-deficient NahG transgenic plants. Number of second stage (J2), third stage (J3) and fourth stage juvenile females (J4) of *H. schachtii* were counted at 14 dpi. Asterisks indicate significant difference in number of nematodes of each developmental stage compared to the wild type Col-0 plants, using Mann-Whitney U non-parametric test. (* = $p<0.05$; ** = $p\leq0.01$, *** = $p<0.05$ and **** $p<0.001$).

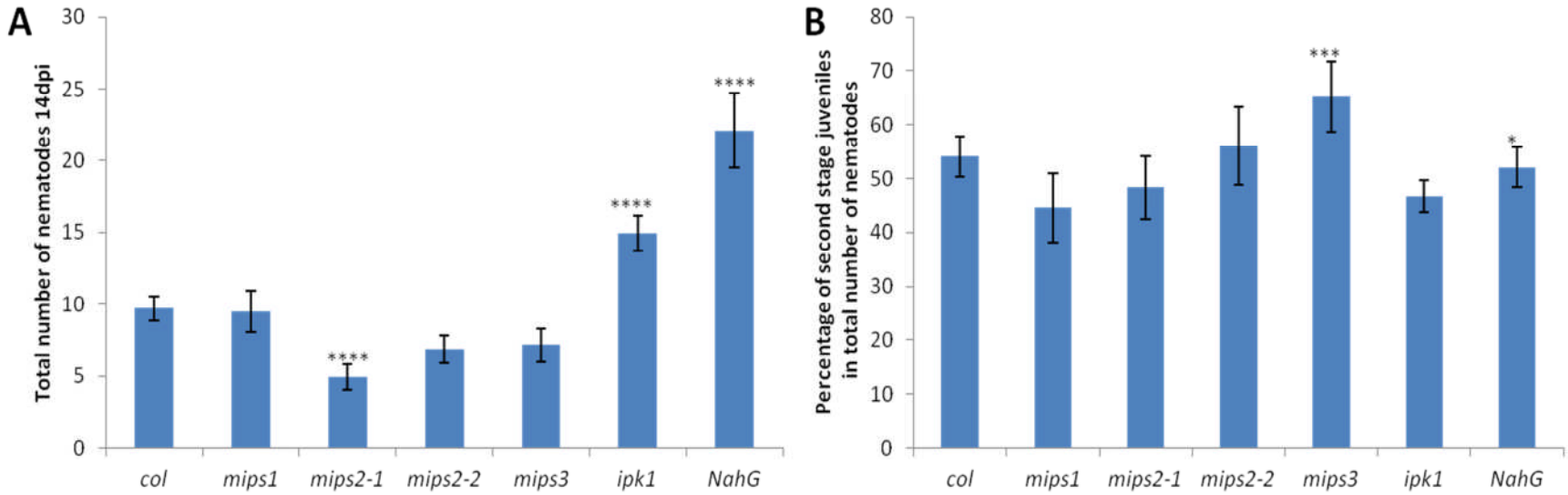


Figure 6-6. Infection assay for low phytate mutants compared to wild type col and salicylic acid NahG transgenic plants infected with *H. schachtii*. A. Total number of *H. schachtii* nematodes per plant. **B.** Proportion of second stage juveniles in total number of nematodes present in infected roots. The mutant and transgenic plants were compared to the control, using Mann-Whitney U non-parametric test. (*significant at $p \leq 0.05$, *** significant at $p \leq 0.005$ and **** significant at $p \leq 0.001$).

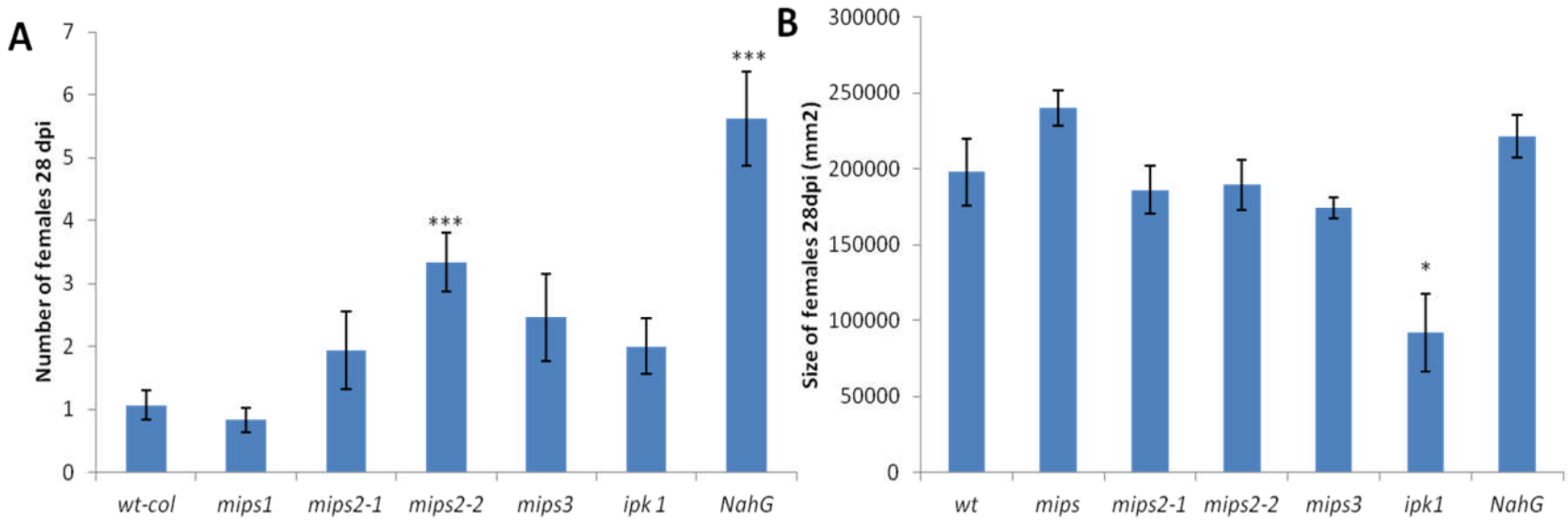


Figure 6-7. Infection assay and size of *H. schachtii*, 28pdi on low phytate mutants compared to wild type col and salicylic acid NahG transgenic plants. A. Mean number of *H. schachtii* females per plant. **B**. Mean size of female *H. schachtii* per genotype. The mutant and transgenic plants were compared to the wildtype, using Mann-Whitney U non-parametric test. (*significant at $p \leq 0.05$ and ***significant at $p \leq 0.005$).

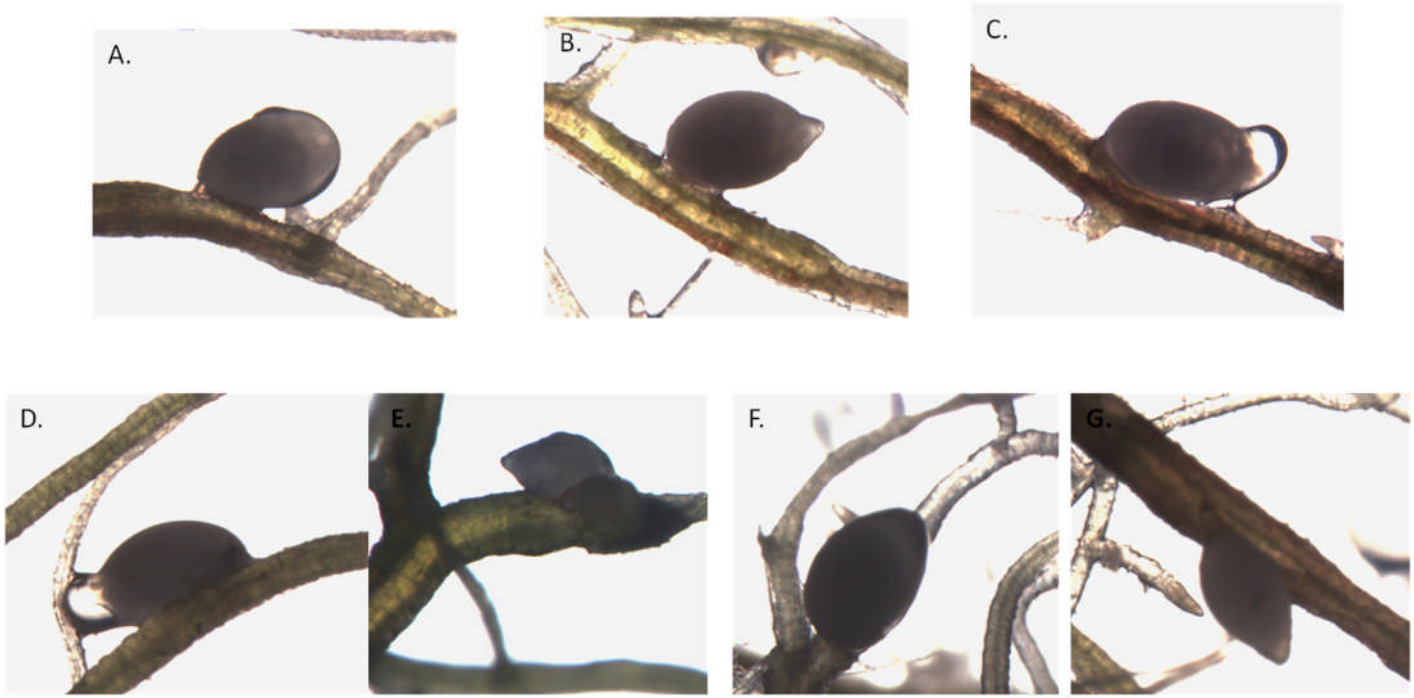


Figure 6-8. Female *H. schachtii* individuals on wild type Col-0 and mutant Arabidopsis plants, 28dpi

Representative *H. schachtii* females on wild type, transgenic and mutant Arabidopsis after 28 dpi - **A.** On wild type Col-0, **B.** on *mips1* mutant, **C.** on *mips2-1* mutant, **D.** on *mips2-2* mutant, **E.** on *mips3* mutant, **F.** on *ipk1* mutant and **G.** on *NahG* transgenic plant. The size was measured using Image pro 7 analyser and mean size of females on the mutant and transgenic plants was compared to the size of females on the wildtype plant, using Mann-Whitney U non-parametric test. ($p \leq 0.05$)

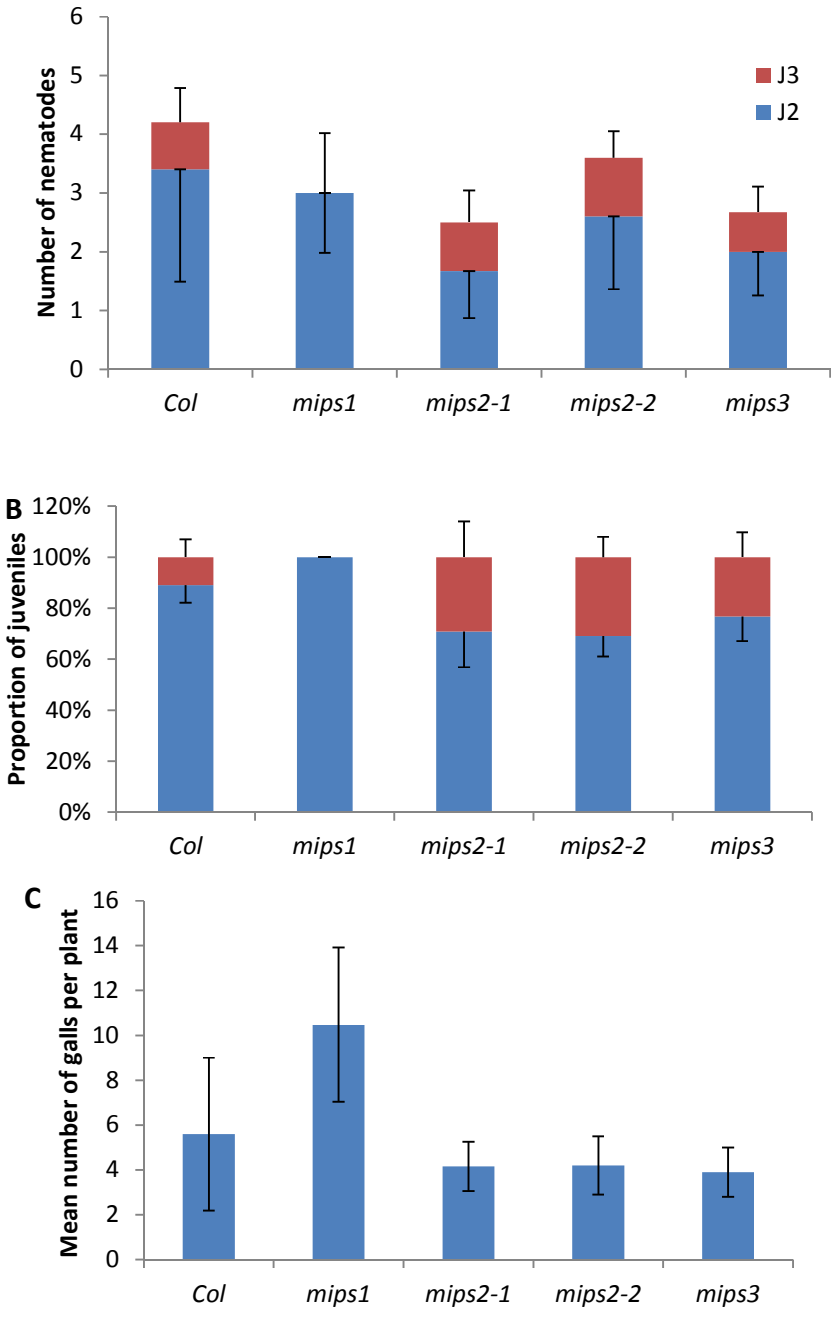


Figure 6-9. Infection assay for low phytate mutant plants compared to wild type Arabidopsis plants infected with *Meloidogyne. graminicola*. A. Mean number of second stage and third stage juveniles present per plant at 14 dpi. **B.** Proportion of second stage and third stage juveniles in total number of juveniles per plant. **C.** Mean number of galls seen per root system at 14 dpi. Non-parametric, Mann-Whitney U test was performed, none of the mutants showed significant difference in susceptibility from the wild type Col-0 Arabidopsis plants.

6.4. Discussion

Growth analysis and nematode infection assays were performed on low phytate Arabidopsis mutants generated by T-DNA insertion in the genes producing the first and last enzymes of the phytate biosynthesis pathway. The *ipk1* Arabidopsis mutant and *NahG* transgenic line displayed delayed development in early growth stages compared to wild type plants. The *MIPS* gene Arabidopsis mutants showed no significant deviation in growth patterns from the wild type plants. Knock-out mutants for the gene producing the IPK enzyme, in which only the level of phytate in cells is reduced without altering *myo*-inositol levels, grow similarly to wild type suggesting that reduced phytate levels do not alter growth characteristics of a plant. A quarter of *mips1* mutant plants exhibited an abnormal phenotype. They showed abnormal seedling growth and had small cotyledons with irregular boundaries and lesions. The phenotype was rescued in later growth stages and mutant plant development was then similar to wild type plants. Meng *et al.* (2009) have reported a similar phenotype for these plants. They showed that the function of *MIPS* genes was non-redundant and *mips1* mutants show abnormal seedling growth. The lesions were presumed to be associated with a hyper-sensitivity response and not programmed cell death and were the result of variable duration and intensity of the light. The role of the *MIPS1* gene in causing phenotypic variation during embryogenesis is controversial. Luo *et al*. (2011) determined the role of *MIPS1* in auxin-dependent embryogenesis but did not report any phenotypic variation in *mips1* mutants when compared to wild type Arabidopsis plants. The *MIPS* genes are developmentally and spatially regulated. *MIPS1* plays a significant role in synthesis of *myo*-inositol during embryogenesis and is highly expressed throughout all embryonic stages. Expression of *MIPS2* and *MIPS3* are restricted to seed coat, maternal tissues and in very low concentrations in embryo and endosperm (Luo *et al.* 2011). Similar expression is observed in other plants like common beans and soybeans (Chiera and Grabau 2007; Abid *et al.* 2011). In a 7 day old Arabidopsis seedling expression of *MIPS1* is highest in the edges of the cotyledons, developing leaf primordia, root tips and the junction of the root and hypocotyl. Whereas *MIPS2* is localised to the hypocotyl, tips of leaf primordia and stipules and *MIPS3* is highly expressed in shoot vascular tissue, hydathodes, trichomes and in root vascular tissue specifically near lateral roots. In a 18-day old seedling *MIPS1* is expressed in all tissue types throughout the plant, expression of *MIPS2* is highest in flowers and siliques and *MIPS3* is strongly expressed

in roots (Donahue *et al.* 2010). The disruption of *MIPS* genes has a more widespread impact on plant growth than disruption of the *IPK* gene. These genes are control points in the production of many vital derivatives involved in various cellular processes.

Low phytate Arabidopsis mutants were also tested to determine their nematode susceptibility. The *ipk1* mutant that has 70 % reduced phytate when compared to wild-type showed increased susceptibility to *H. schachtii* infection. These mutants are also significantly more susceptible to viral, bacterial and fungal pathogens (Murphy *et al.* 2008). This study showed that *ipk1* mutants have no defect in germination, plant development or the seed yield of the plants. In spite of a reduction in phytate level the total phosphorus content of seeds is not reduced and they did not show accumulation of phytate precursors (Stevenson-Paulik *et al.* 2005). This has suggested that disruption of the *IPK1* gene is an excellent strategy for development of agricultural and nutritionally improved low phytate crops. From the present study however, it appears that substantial reduction in seedling phytate level by disruption of the *IPK1* gene leads to enhanced nematode susceptibility. *ipk1* mutants show infection levels similar to a salicylic acid deficient *NahG* transgenic line that does not have functional systemic acquired resistance, and supports a high level of nematode development (Wubben, Jin and Baum 2008). The *ipk1* mutants have also shown enhanced susceptibility to viral, bacterial and fungal pathogens, suggesting that although reduction in phytate level is an excellent strategy for agricultural and nutritional purposes it can lead to generation of plants which are highly susceptible to biotic stress, which in turn will lead to higher yield loss.

Both *mips1* and *mips2-1* mutants show 60% reduction of total phytate content but have antagonistic responses to pathogen attack. The *mips1* mutants showed no deviation from wild type pathogen response in the case of nematodes and other microbial pathogens. The *mips2-1* mutants show reduced susceptibility to *H. schachtii* but in earlier studies *mips2-1* mutants have shown increased susceptibility to microbial pathogens (Murphy *et al.* 2008). The *mips2-2* mutants (no data available on phytate content in seeds or seedlings) show a response similar to *mips1* mutants and have no deviation from wild type pathogen response. Although both *mips2-1* and *mips2-2* mutants are knockouts for the *MIPS2* gene, the *mips2-1* mutant has an increased level of *MIPS1* which makes it an over expression mutant of the *MIPS1* gene and a knockout for *MIPS2* (Donahue *et al.* 2010). It is very likely that the antagonistic response seen in *mips1* and *mips2-1* mutants towards cyst nematode susceptibility is an outcome of difference in expression levels of

MIPS1. Literature suggests that the majority of MIPS proteins are cytosolic in nature with very small amounts in organelles, cell walls and membrane structures but still the sub-cellular localisation of MIPS proteins is not very clear (Mitsuhashi *et al.* 2008). A widely accepted hypothesis suggests the presence of spatially separated pools of phytate resulting from each isoform of MIPS enzyme (Murphy *et al.* 2008). The *mips3* mutants harbour a higher proportion of second stage juvenile *H. schachtii* at 14 dpi, suggesting that they do not support healthy development of nematodes. No data is available on phytate content and response of these mutants towards other microbial pathogens. *mips3* mutant plants are knocked out for *MIPS3* and have normal levels of *MIPS1* and *MIPS2* (Donahue *et al.* 2010).

The finding that *mips2-1* and *mips3* mutant plants are less susceptible to cyst nematode infection was unexpected, but may be explained by considering the changes that are necessary in the plant to allow a successful infection to establish. Since the plant parasitic cyst nematode establishes a feeding site in the root vascular bundle of plant, reduced levels of vascular tissue-specific *MIPS3* might have a major effect on establishing a nematode feeding site and will reduce susceptibility of these mutants towards nematode infection. An increased level of inositol phosphate and its derivatives in syncytia highlights the importance of *MIPS* genes in establishment of a feeding site (Siddique *et al.* 2009). The low phytate rice developed by RNAi silencing of the *MIPS* gene shows increased sensitivity to ABA, which in turn can affect the defence response of a plant to biotic pathogens (Ali *et al.* 2013a).

During a compatible cyst nematode infection the second stage juvenile initiates formation of an enlarged, multinuclear nutrient sink called a syncytium (Sharma 1998). Formation of syncytia requires cell wall remodelling and strengthening to prevent the cell from collapsing under enormous turgor pressure of 9000-10000 hPa (Böckenhoff and Grundler 1994). This process requires synthesis of polysaccharides derived from a cell wall-specific biochemical precursor UDP-glucuronic acid (Klinghammer and Tenhaken 2007). UDP-GlcA can be produced by two alternative pathways. The first includes dehydrogenation of UDP-glucose by the enzyme UDP-glucose dehydrogenase (UGD). The alternative pathway involves a family of MIOX enzymes that catalyses conversion of *myo*-inositol to glucuronic acid, which is later converted to UDP-glucuronic acid (UDP-GlcA). During a compatible cyst nematode infection the levels of *MIOX* gene expression increase dramatically in syncytia. There are a total of four *MIOX*

genes identified in Arabidopsis; *MIOX1, MIOX2, MIOX4* and *MIOX5, MIOX 3* is a pseudo-gene. In uninfected plants *MIOX4* and *MIOX5* are strongly expressed specifically in pollen, *MIOX2* is expressed in roots and throughout the seedling. The *MIOX* genes in Arabidopsis are functionally redundant, knock-out mutants for single *MIOX* genes do not show a significant difference in nematode infection levels as compared to wild type, the double *MIOX* mutants supported a reduced number of female nematodes along with poorly developed and small syncytia (Siddique *et al.* 2009). Cell wall component analysis of uninfected *miox1/2/4/5* mutants indicates 90 % inhibition in incorporation of *myo*-inositol derived sugars. Complete blockage of the MIOX pathway in the quadruple *MIOX* knockout is likely to push the cell towards the alternative UGD pathway, which might not happen in double *MIOX* mutants or in the presence of all *MIOX* genes (Endres and Tenhaken 2011). This is also supported by up-regulation of a UDP-xylose synthase gene in syncytia which possibly blocks UDP-GlcA pathways in infected plants due to feedback inhibition of UGD caused by accumulation of UDP-xylose (Klinghammer and Tenhaken 2007). Increased levels of MIPS isoforms and various enzymes required for UDP-GlcA synthesis in the syncytia strengthen the role of MIOX pathways as the main UDP-GlcA synthesis pathway (Jammes *et al.* 2005; Siddique *et al.* 2009). We propose that a compatible nematode interaction requires excess cell wall polysaccharides. Reduced expression of *MIPS* leads to less *myo*-inositol and less cell wall polysaccharide production by the MIOX specific pathway, the UGD pathway is blocked by negative feedback due to increased UDP-xylose and thus cyst nematodes fail to establish a healthy feeding site. These results suggest that *myo*-inositol derivatives are also required for healthy development of cyst nematodes and syncytia.

In compatible root-knot-nematode infections, the second stage juveniles initiate a feeding site, known as a giant cell complex, near the zone of differentiation. Giant cells are enlarged cells, formed by repeated rounds of mitosis without cytokinesis (Bird, Opperman and Williamson 2009). There was no significant difference observed in susceptibility of *mips* mutants towards root-knot-nematodes. *MIOX:GUS* reporter lines infected with *M. incognita* juveniles do show an increased expression of *MIOX2, MIOX4* and *MIOX5* in gall cells. The GUS expression was not localised to galls and was spread throughout the roots (Atkins 2009). However, expression analyses of giant cells show very weak differential expression of *MIOX* and *MIPS* genes under RKN infection (Jammes *et al.* 2005; Damiani *et al.* 2012). No data is available on the effect of

MIOX on nematode development or giant cell formation. The results obtained suggest *myo*-inositol derivatives do not have a significant role in RKN infection and formation of giant cells.

6.5. Conclusion

This study has shown that a reduced level of phytate does not massively affect the growth characteristics of the mutant Arabidopsis plants. Disruption of the *IPK* gene reduces basal resistance of the plant and makes it highly susceptible to nematode infection. The *mips1*and *mips2-1* mutant plants have a similar rate of cyst nematode infection as wild type Arabidopsis plants. The *mips2-2* and *mips3* mutant plants have lower infection than the wild type plants. Low phytate content in plants due to disruption of any of the *MIPS* genes has no effect on root-knot-nematode infection. The results indicate that some routes to development of low phytate plants for the environmental and nutritional benefit can introduce challenging pest problems due to reduced basal resistance of the low phytate plants (Raboy 2001).

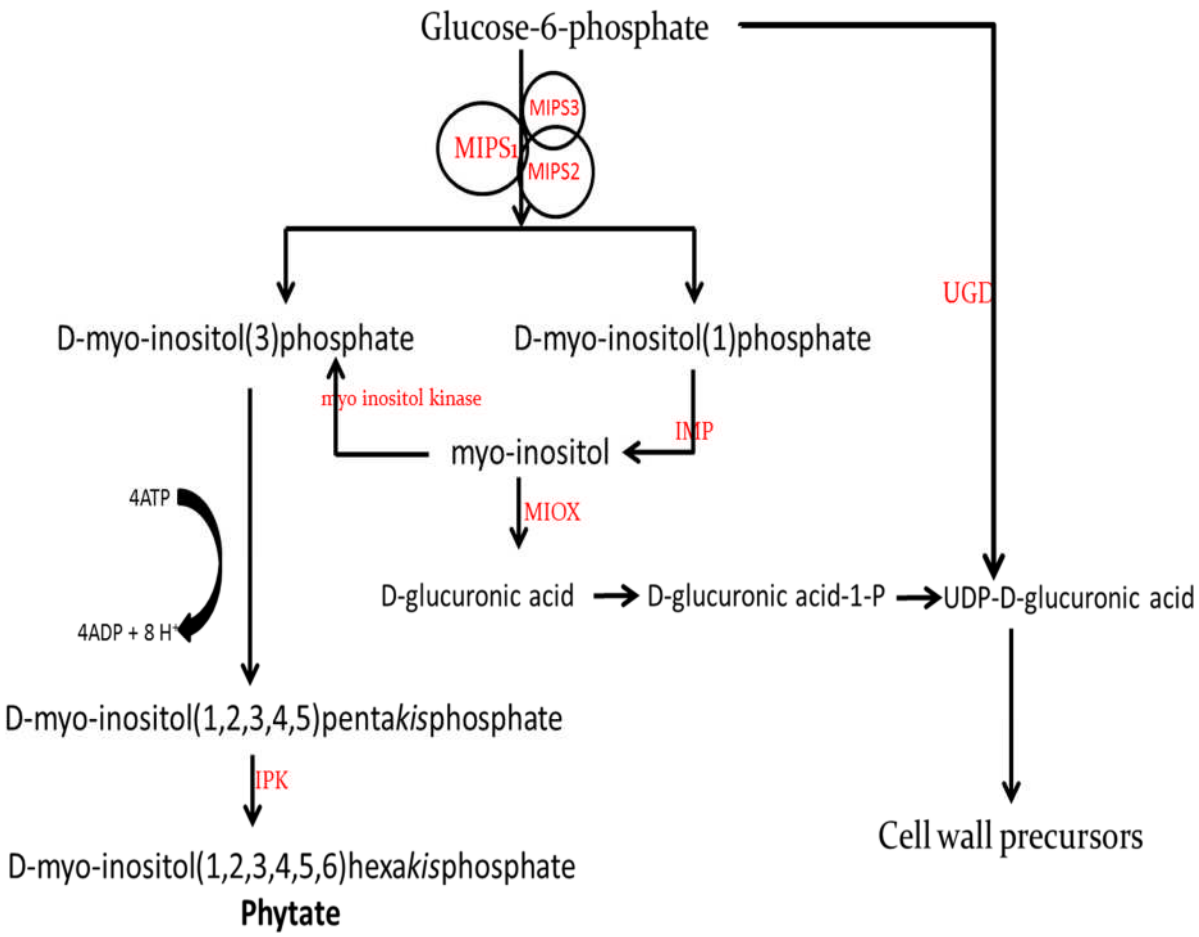


Figure 6-10 – Schematic representation of phytate synthesis

Glucose-6-phoshate is converted to *myo*-inositol phosphate in a rate limiting reaction catalysed by *myo*-inositol phosphate synthase (MIPS). *Myo*-inositol goes through a series of phosphorylation steps to yield phytate. The last step of phytate synthesis is catalysed by inositol polyphosphatasekinase (IPK1). Another important derivative of *myo*-inositol is UDP-glucuronic acid which is a precursor for cell wall polysaccharides. These polysaccharides are important for strengthening of the syncytial cell wall in cyst nematode infection. *Myo*-inositol oxygenase (MIOX) enzyme catalyses the reaction converting *myo*-inositol to glucuronic acid. This is further converted to UDP-glucuronic acid. An alternative pathway of production of UDP-glucuronic acid by direct conversion from glucose-6-phosphate is catalysed by UDP-glucose dehydrogenase. Upregulation of *MIOX* expression in syncytia and negative inhibition of the UGD pathway by accumulation of UDP-xylose suggest that MIOX is the main cell wall precursor-producing pathway in infected roots and limiting *myo*-inositol production in *MIPS* mutants limit this pathways and leads to reduced nematode susceptibility.

7 Chapter

General Discussion

This study has aimed to identify the transcriptomic response of rice plants towards simultaneous drought and RKN infection by performing a microarray analysis using the rice genome chip. A laboratory based realistic model was designed to mimic simultaneous stress conditions as experienced by the plants in the field. The study was accompanied by a field-based study to validate the effect of combination of simultaneous stress on the rice plants under natural conditions.

7.1. Relevance of crop plants in investigating plant stress responses

With the changing climate and growing population, the agricultural reforms in the twentieth century necessitate development of newer crop varieties that can tolerant the rickety climate changes and at the same time can produce high quantities of nutritious food grains. In the last two decades, plant research has focused on exploring stress responses and identification of molecular mechanisms that can be useful in manipulating the plant response towards environmental stresses. The present scenario calls for use of a model crop plant like rice in determining the molecular responses towards environmental stresses and for development of novel technologies that can have direct impact on quality and quantity of global agriculture globally. This study has successfully used rice as the model plant to explore the plant responses towards drought and RKN infection and identify candidate genes in a crop species that can possibly be used as targets for yield improvement under stressed conditions. The loss of function mutation in the peroxidise precursor gene identified in our study results in improved performance and stress tolerance in rice plants grown under realistic simultaneous stress conditions as well as in unstressed conditions. This sets an example that the research conducted using crop plants can lead to direct identification of target genes that can be utilised to improve plant performance in field conditions. Although, the contribution of Arabidopsis in plant molecular research has been unprecedented, successful transfer of technologies developed in Arabidopsis into higher crop plants have been difficult and a time consuming process. A large number of genes has been identified as stress responsive in Arabidopsis and out of these just a few have been used to impart stress tolerance in crop plants (Peleg, Apse and Blumwald 2011; Aleksandra *et al.* 2011).

Most of the rice genes do not have an orthologue in Arabidopsis which further limits relevance of the discoveries made in Arabidopsis (Salse *et al.* 2002; Rensink and Buell 2004). Arabidopsis is not a natural host of plant parasitic nematodes. Although, the CN and RKN are capable of completing their lifecycle on Arabidospsis, its use in understanding of plant-nematode interaction is limited. No resistance gene against plant parasitic nematodes has been identified in Arabidopsis as of yet, this restricts the comparison of compatible and incompatible interactions (Gheysen and Fenoll 2011). The host-specific nature of many plant nematode interactions further limits use of Arabidopsis. Although rice has longer generation time (3-4 months) and is difficult to manage in controlled growth chambers when compared to Arabidopsis, it is encouragingly being accepted as the major model monocot plant. In field studies, research is severely limited to the rice growing season (Ohnishi *et al.* 2011). But a sequenced genome along with a plethora of bioinformatic tools that can be used for investigation and genomic manipulation techniques mean that use of Arabidopsis in the stress responsive studies and transgenic studies has slowly decreased and the role is being taken over by model crop species like rice (Tester and Bacic 2005).

7.2. The need for a realistic stress model in molecular studies

This study has proved that growing rice in soil under a realistic stress model to evaluate yield parameters in controlled growth chambers is a challenging but rewarding process. In spite of all the challenges and the difficulties, the use of fully-grown rice plants cultivated in soil helps in better understanding of the morpho-physiological effects of the stress on the plant growth and yield parameters. The important aspect of crop stress research is to enhance the crop yield and determine the effect of stress on the nutritional content of the produce. Such a system straight away determines the effect of the imposed stress on these important parameters and at the same time mimics the natural stress and helps in understanding plant response to the natural stress.

The nature of stress experienced by plants in the fields is different from the stress imposed in laboratory. Most of the transgenic plants that claim to accomplish improved stress tolerance are screened in the laboratory or glasshouse under severe stress. Use of unrealistic approaches of imposing stress like air-drying, dehydration, artificial infection in tissue culture and exogenous application of hormones are used to identify the target stress responsive genes. Thus the transgenic studies based on these target genes often

fail to accomplish the claimed success in crop species. Even in the model plant Arabidopsis, the stress tolerance genes identified on the basis of a survival test following a prolonged period of complete soil dryness respond differently to the gradual stress. It is established that the increased survival rate is just a result of improved water usage and does not always result in improved plant reproduction. (Aleksandra *et al.* 2011). This might be a reason for the low rate of success in transferring the laboratory knowledge to the fields Thus, it is necessary to develop realistic model systems that efficiently mimic stress to identify the key players of the field stress response in crop plants.

Due to the long generation time and size of the mature rice plants, researchers have substituted the need of using soil grown mature rice plants by hydroponic cultivation system and the use of seedlings (Ohnishi *et al.* 2011). As routine agronomic practice, rice is germinated in nurseries, cultivated there for first few weeks, and then transplanted into the fields to assure that the seedlings are not subjected to any major stresses (DRD 200). The laboratory models that use seedlings and hydroponics fail to mimic the realistic stress situations endured by plants. Using mature plants for the molecular studies increases another level of complexity. It provides an opportunity to compare changes in different leaf types and different parts of root system to understand the role of senescence and aging, at same time it also facilitates study of reproductive system in stress responses. It gives the researcher an opportunity to ask bold questions and design experiments to look at more specific plant tissue responses rather than just the vegetative response. Nematode mobility is dependent on soil moisture content and pore size, and these variables significantly influence the rate of infection (Section 2.3.7)(Prot and Van Gundy 1981). The study carried out in a soil-based system efficiently mimics the environment nematodes will come across in fields and gives a realistic estimate of infection rate compared to tissue culture / growth pouch based infection studies. Moreover, use of mature plants evaluates the effect of nematode stress on the plant yield and not just on plant growth. In the case of combined drought and nematode treatment, use of mature plants correctly mimics the vegetative stress condition as it would be encountered in the fields. The developed model mimics the conditions faced by rice plants under aerobic cultivation. Use of crop plants under realistic conditions for studying the response of various environmental factors is the way forward for agricultural research.

7.3. Simultaneous multiple stresses induce specific suite of genes

This study showed that the plant response to simultaneous drought and nematode stress was dominated by the drought-specific response but also had transcriptomic changes that were very specific to the simultaneous stress only. Considering the semi-aquatic nature of lowland rice cultivation, water scarcity can severely impair the physiological functions and growth in rice. When it is accompanied by an equally challenging biotic stress, like the RKN infection, that is capable of altering plant cellular function, the resultant response is interactive rather than additive. During gradual vegetative drought, flowering was delayed and less total body mass was produced. The limited amount of water available was used for maintaining the minimum cellular function for survival. This severely limits yield. During moderate RKN infection in this study total plant biomass increased, more tillers are formed but the yield was not significantly affected. In the fields, successive cropping and/or multiple nematode generations on the host plant can cause nematode populations to increase to an alarming level. However, the drought stressed plants that were simultaneously stressed with RKN in this study had a lower level of nematode infection than the plants infected with same number of nematodes under well-watered conditions. It is difficult to conclude if the reduced nematode infection was because the lower soil moisture limited the number of nematodes entering the plant or because reduced availability of resources to the giant cells limited the nematode growth and reproduction. Reduced nematode infection may also be attributed to a smaller root system in drought stressed plants. The water relation in multiple stressed plants was improved with higher fresh shoot weight with reduced total biomass but the yield was drastically reduced. This suggests that out of the two, drought is a more devastating stress and dominates the plant's response towards simultaneous drought and RKN infection. A similar picture was seen in the transcriptomic analysis; most of the changes in the multiple stressed plants were also seen in drought stressed plants. The RKN infestation induced gene expression changes of a very low magnitude, but these changes were nevertheless sufficient to initiate the unique molecular response in the multiple stress treatment. The multiple stress response was characterised by the gene families involved in hormonal cross talk and signalling cascades indicating strong cross talk between the two stresses. A metabolic study conducted to investigate the effect of drought and nematode stress on the metabolite content of tomato fruits saw an increase in the fructose and glucose levels in only in the

multiple stressed plants (Atkinson *et al.* 2011). The up-regulation of genes related to sugar biosynthesis in this study could result in similar increase in sugar concentration in rice.

The results from the transcriptomic study suggest that different members of certain gene families can respond to different stresses and mediate a stress response. Gene families like LTPLs, cytochrome P450, dirigents, nodulins and receptor kinases stand out for their differential roles in biotic and abiotic stresses.

It is now becoming widely accepted that the plant response to a combination of simultaneous stresses has recently taken the centre place in plant research. Previously, information obtained from experiments investigating the effect of different stresses individually was used to predict the plant response to multiple stresses. Similarly, transcriptomic data from stress studies and mutant studies have been used to identify the crosstalk between biotic and abiotic stresses (Fujita *et al.* 2006; Sharma *et al.* 2013; Narusaka *et al.* 2004; Baena-González *et al.* 2007; Grennan 2006). Lately, it has been accepted that individual stresses suffered by a plant may act in combination to produce additive, interactive or antagonistic effect . The plant response is tailored specifically to combinational stresses and thus each pair of combinatorial stress pairs needs to be explored in a plant by simultaneous implication of the stresses.

7.4. Plants respond differently to a set of stresses in field compared to the same stresses imposed in controlled environment

In this study, the laboratory based microarray analysis was accompanied by a field study of an indica and a japonica low land rice cultivar under the simultaneous drought and nematode stress. The field study showed similar growth and yield responses towards simultaneous stress but the effect on yield was less severe than that observed in the laboratory study. The field study also confirmed that the commercially accepted lowland indica cultivar and lowland japonica cultivar, commonly used in the laboratory studies, responds similarly to the simultaneous drought and nematode stress. The lowland cultivars, of both sub-species, respond to simultaneous biotic and abiotic stresses in similar fashion. The molecular analysis reveal that the hormonal interplay in the plants grown in fields is more complicated than predicted by the laboratory studies.

Comparison of the field study and the laboratory based stress treatment has revealed that the gradual vegetative stress induced by 50 % reduction in soil moisture content in

a pot-based experiment has severe repercussions on the yield of the plant when compared to the gradual vegetative stress seen in the fields. Laboratory studies performed on transgenic seedlings usually focus on the survival of plants under extreme stress conditions but fail to assess the effect on subsequent plant development and yield. Transgenic rice over-expressing *APETALA2 (AP2)* gene *AP59* exhibits drought and salt tolerance in laboratory studies but suffers 23 % - 43 % yield loss in the field (Oh *et al.* 2009).

Conducting a field study can be a daunting task without sufficient knowledge of agricultural practices as the planning and organisation is completely different from that of a laboratory experiment. Especially for a study of this nature, it is vital to get a levelled field that has uniform soil composition to prevent differential drying during the stress treatment. At the same time, weeds, pests and pathogens have to be checked regularly. Use of chemical pesticides and herbicides can initiate molecular responses in the target crop specie that can interfere with the stress responsive molecular mechanism under investigation. Depending upon the nature of the soil, plants may require a custom fertilisation program unlike the standard fertilisation done in the laboratory experiments. Nutrient deficiency can mimic symptoms similar to the biotic and abiotic stresses and mislead the results. Phosphorus deficiency in rice can lead to a reduced *Fm/Fv* ratio similar to the reduction seen under biotic stresses (Xu, Weng and Yang 2007). At the same time, nutrient deficient soil can manifest the effects of otherwise less severe abiotic stress. as example, reduced K levels in soil can increase leaf rolling and reduce leaf conductance when compared to drought stressed plants with optimal K fertilisation (Premachandra *et al.* 1993). Similarly ammonium nutrition can also affect rice response to drought stress (Li *et al.* 2009b).

Although, it is desirable to explore plant response to stresses in a realistic way, obtaining the stringency and consistency required for transcriptomic and molecular studies in the field is a difficult task. In the fields, there are a large number of variables that cannot be accurately monitored. Field studies fail to provide significant molecular resolution in response to a single stress because of numerous variables. Thus, field studies cannot surpass the need of laboratory-based experiments. But lessons needs to be learnt from the sparse successes of stress tolerant varieties developed in the laboratory to ascertain the importance of supplementing the laboratory experiments with

field studies and field stress screenings (Atkinson and Urwin 2012; Deikman, Petracek and Heard 2012; Mittler 2006).

7.5. The order in which stresses occur alters plant response

The response of a plant towards a pair of a biotic and an abiotic stresses can depend on the timing, nature and severity of the stresses under consideration. The transcriptomic changes in the simultaneously drought and nematode stressed plants were more similar to the drought stress specific changes than to the nematode specific changes. However, the RKN infection on drought stressed plants did not have an additive effect, as seen in a similar study on Arabidopsis under CN and drought stress (Atkinson 2011). Similar additive effect of drought stress, induced by osmotic arrangements caused by infection of an endophyte was seen on the reproduction of *M. marylandi* in tall fescue plants (Elmi *et al.* 2000). It suggests that the severe drought stress can lead to reduced RKN multiplication in rice. The simultaneously dual stressed plants performed better physiologically than the plants that were only drought stressed. Similar transcriptomic responses were observed in Arabidopsis plants simultaneously stressed by cyst nematode (*H. schachtii*) and dehydration imposed in a tissue culture based study. An additive effect of two stresses was seen. However, the author saw increased nematode susceptibility in drought stressed plants concluding that when under simultaneous biotic and abiotic stresses, the plant response are dominated by the more severe abiotic stress (Atkinson, Lilley and Urwin 2013). A field study investigating the effect of cyst nematode infection and drought in upland rice has also shown an additive effect of the two stresses with the simultaneously stressed plants suffering a more severe yield losses (Audebert *et al.* 2000). We observed an interactive response towards drought and RKN infection in pots as well as the field study. The combination of drought and nematode stress had an additive effect on the photosynthetic apparatus of both cultivars. Both drought and nematode infection can cause damage to the photosynthetic apparatus leading to reduction in chlorophyll content and reduction in maximum quantum yield of the photosystem II and the effect was enhanced when both stresses were applied simultaneously (Oh *et al.* 2009; Audebert *et al.* 2000). However, interestingly, when the plants heavily infected with cyst nematodes were drought stressed, the plant water relation was improved with higher fresh and dry weight been produced in both cultivars. The reduction in yield was also less than for the plants that were only drought stressed.

Considering that in the field study plants were infected with nematode prior to drought stress, unlike the laboratory study, it suggests that the order in which the two stresses were imposed can change the plant responses. Thus, it could suggest that if plants are under attack of a severe biotic stress, the damage caused by drought is not so severe. Nematodes are capable of improving plant water relation that can in turn moderates drought manifested physiological changes in plants. The Cyst nematode, *Globodera pallida* is known to reduce transpiration rate in the potato plants and *H. sacchari* reduces stomatal conductance in rice (Schans 1991). Similarly other microbial pathogens are also known to reduce plant water loss by initiating stomata closure and other physiological changes similar to drought avoidance mechanism adopted by plants (Beattie 2011). Drought stress in plants increases the concentration of defence compounds due to less available water, this increases plant susceptibility towards pathogens. Concentration dependent ABA mediated resistance to powdery mildew is seen in Barley when the plants are subjected to abiotic stress in hydroponic cultures (Wiese, Kranz and Schubert 2004; Atkinson and Urwin 2012). Another study also established that root herbivores can enhance resistance to leaf herbivores by reduction in leaf water potential due to ABA mediated increase in defence compounds (Erb *et al.* 2011). In the pot study, occurrence of severe drought before nematode infection may have enhanced the concentration of defence compounds or initiated the ABA mediated stress response and thus reducing the nematode multiplication. The changes in growth and yield parameters observed in the plants under simultaneous stress indicate that severe drought can reduce nematode infection and the severe nematode infection can limit the damage caused by the drought. These results can also be attributed to the fact that the cyst nematode and RKN have different modes of infection. Unfortunately, a CN control treatment was not included in the study because of limited CN infested field space. Presence of a positive control for CN would have otherwise strengthened our findings. In the light of current results, we propose that the plant prioritises its response to severe stress irrespective of it being biotic or abiotic and the order in which stresses initiate in the plant plays an important role in determining the total response towards a pair of simultaneous stresses.

7.6. Manipulating nutrition component of crop plants can affect stress responses

A part of this study, analysing transgenic Arabidopsis that have a reduced level of anti-nutrient phytate, determined that reduction of phytate in the plant could lead to increased susceptibility to pathogens including PPN. Various steps of phytate biosynthesis in plants have been targeted to reduce the phytate content of the plant and the produced grains. This study focus on transgenic plant with targeted manipulation of two major steps (Chapter 6). The loss-of-function mutation in *IPK* gene in Arabidopsis definitely leads to reduced plant basal defence. Phytate level has been successfully reduced in crop species including rice, soybean and maize by manipulation of the *IPK* gene (Shukla *et al.* 2009; Yuan *et al.* 2012; Raboy 2001). The effect of reduced phytate level on the basal resistance in these crop species has not been analysed. However, novel strategies are employed to manipulate *IPK* expression only in the edible part of the crop, to avoid penalty caused by reduced total phytate content of a plant. In rice, seed-specific RNAi-mediated silencing of the *IPK* gene leads to 3.85-folds reduction in the phytate content and 1.8-fold increase in accumulated iron content with no penalty on plant growth and yield (Ali *et al.* 2013b). At the same time, other gene targets have been exploited to reduce the phytate content (Xu *et al.* 2009; Zhao *et al.* 2013; Kuwano *et al.* 2009b; Cichy and Raboy 2009). Phytate holds an imperative role in human nutrition, apart from being a anti-nutrient it also possesses therapeutic properties and thus the correct dosage of phytate inq3 human food is critically important (Kumar *et al.* 2010). In addition to phytate, other plant nutrients also influence the defence response of a plant and the change in a plant defence response needs to be considered in the process of improving nutritive value of the crop species. This study shows a negative effect of manipulated nutrient content on plant defence although other nutrients can have a positive effect as well. Biofortification of vitamin C in tomato imparts nutritional benefits in humans and at same time increases plant defence against diseases resulting in better yield (Locato, Cimini and De Gara 2013).

Although plant research has always prioritised yield improvement over nutrition, recently there has been a frame shift (Raboy 2013). Development of low phytate plants to increase bioavailability of other nutrients has been one of the major steps towards achieving more nutritious food accompanied by biofortification. Sustainable agriculture calls for improvement in plant yield as well as the nutrient content of the food. The

stress treatments can alter nutritive content of the plant and *vice versa*. Thus it is important to understand the plant responses towards combination of biotic and abiotic stresses and also to evaluate how modification in the nutritive content of crop plants will change these responses.

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List of Abbreviations

ABA	Abscisic Acid
ANOVA	Analysis of Variance
BLAST	Basic Local Algorithmic Search Tool
BR	Brassinoids
Ca^{2+}	Calcium
CaMV	Cauliflower Mosaic Virus
cDNA	Complementary DNA
cm	Centimetre
CN	Cyst Nematode
CO_2	Carbon Dioxide
col	Columbia
CTAB	Cetyl trimethylammonium bromide
DEG	Differentially expressed genes
DNA	Deoxyribonucleic acid
Dpi	Days post infeciton
DSW	Dry shoot weight
EDTA	Ethylenediaminetetraacetic acid
EST	Expressed sequence tag
ET	Ethylene
FACE	Free-Air CO2 Enrichment
FC	Field Capacity
FDR	False discovery rate
FRW	Fresh root weight
FSW	Fresh shoot weight
GFP	Green fluorescent protein
GO	Gene Ontology
GUS	β-glucuronidase
ha	Hectare
Hr	Hour
ID	Identities
IP	Inorganic Phosphate
ISC	Initial syncytium cell
J2	Second stage juvenile
J3	Third stage juvenile
J4	Fourth stage juvenile
JA	Jasmonic Acid
K^+	Potassium
kg	Kilogram
LB	Luria-Bertani medium
LOC	Locus
m	Metre
Mbp	Million base pairs
mg	Milligram

min	Minute
nol	Mole
MS media	Murashige and Skoog media
MSU	Michigan state university Id
N	Nitrogen
nM	Nano molar
PAMP	Pathogen Associated Molecular Patterns
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PK	Protein Kinase
PPN	Plant Parasitic nematodes
PPNEMA	Plant Parasitic nematodes multialigned ribosomal cistron bioinformatic resource
PR	Pathogenesis related
PTI	Pathogen triggered Immunity
qPCR	Quantitative Polymerase Chain reaction
RAP	The Rice Annotation Project
RAP-DB	The Rice Annotation Project- Database
rDNA	ribosomal DNA
RGAP	Rice Genome Annotation Project
RIN	RNA integrity number
RKN	Root-knot nematode
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcription PCR
RWC	Relative water content
s	second
SA	Salicylic Acid
SAR	Systemically acquired resistance
S-N-K test	Student-Newman-Keuls test
SPAD	Soil plant analysis development
TAE buffer	Tris-acetate-EDTA
T-DNA	Transfer DNA
TF	Transcription factor
UV	Ultraviolet
WT	Wildtype
Zn	Zinc
µM	micro molar