MOLECULAR CHARACTERIZATION OF BLACK PEPPER (*Piper nigrum* L.) GENOTYPES FOR DROUGHT TOLERANCE

By

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(2013-11-191)

THESIS
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DECLARATION

I, hereby declare that the thesis entitled **"Molecular characterization of black pepper genotypes (*Piper nigrum* L.) for drought tolerance"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me any degree, diploma, fellowship or other similar title of any other University or Society.

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Certified that the thesis entitled **"Molecular characterization of black pepper (*Fiper nigrum* L.) genotypes for drought tolerance"** is a bonafide record of research work done independently by **Ms. Pallavi V (2013-11-191)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to her.

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ABBREVIATIONS

A	Adenine
ABA	Abscisic acid
ATPase	Adenine triphosphatase
BLAST	Basic Local Alignment Search Tool
C	Cytosine
^{0}C	Degree Celsius
cm	Centimeter
cDNA	Complementary DNA
CPBMB	Centre for Plant Biotechnology and Molecular Biology
DD-RT-PCR	Differentially Displayed Reverse Transcriptase Polymerase Chain Reaction
DEPC	Diethyl pyro-carbonate
DIC	Distributed Information Centre
DMSO	Dimethyl sulfoxide
DNTP	Deoxy Nucleotide Triphosphate

Ds	Double stranded
E.Coli	*Escherichia coli*
EDTA	Ethylene Diamine Tetra Acetic acid
ESTs	Expressed Sequence Tags
G	Guanine
g	Gram
HSP	Heat shock protein
H_2O_2	Hydrogen peroxide
IPTG	Isopropyl thiogalactose
ISR	Induced systemic resistance
IST	Induced systematic tolerance
KAU	Kerala Agricultural University
Kb	Kilo base
kDa	kilo Dalton
LB	Luria Bertoni
LEA	Late Embryogenesis Abundant
M	Mole

Mg	Milligram
min	Minute
ml	Millilitre
mM	Millimole
M-MuLV RT	Murine-MolodenyLukemia Virus Reverse Transcriptase
MOPS	2-(N-Morpholino) ethanesulphonic acid
mRNA	Messenger RNA
µg	Microgram
µl	Microlitre
µM	Micromole
NAD	Nicotinamide Adenine Diphosphate
NCBI	National Centre for Biotechnology Information
ng	Nanogram
nm	Nanometer
OD	Optical Density
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentrartion

%	Per cent
RNA	Ribo Nucleic Acid
RNase	Ribonuclease
ROS	Reactive Oxygen Species
SDS	Sodium dodecyl sulphate
Sec	Second
T	Thymine
TAE	TrisAcetate EDTA
TBE	Tris Base EDTA
TE	Tris EDTA
TIGR	The Institute for Genomic Research
U	Unit
UTR	Untranslated region
UV	Ultra violet
V	Volts
X-gal	5-bromo 4 chloro 3- indolyl β- D galactosidase

Introduction

1. INTRODUCTION

Black pepper (*Piper nigrum* L.) – 'King of spices' – is one of the oldest spices known and is grown for its spicy berries. It belongs to the family piperaceae. It is a perennial climber. Cultivated *Piper nigrum* is monoecious, having hermaphrodite and protogynos flowers and predominantly self pollinated. *Piper nigrum* is a tetraploid species with basic chromosome number 2n=52 (X=13) (Jose and Sharma, 1984). It originated in the humid, tropical evergreen forests of Western Ghats of India and is now grown in more than 25 countries, particularly in tropics. Important growing locations are India, Indonesia, Malaysia, Brazil, Thailand, Sri Lanka, Vietnam and China.

Pepper is a shade loving plant and needs a support to cling on. The black pepper has a great export potential and the contributory factor is the presence of chemical piperine in it. In India, black pepper is generally grown in homestead gardens as a rainfed crop. Though the average rainfall in pepper growing areas is well above 2000 mm, the distribution is not uniform. The rainfall received during December to May is very negligible. Hence, the crop suffers due to severe soil moisture shortage during these months. Moisture stress which results from withholding water supply leads directly to changes in the physical environment of the crops that subsequently affect physiological and biochemical processes. Literature regarding the impact of water stress on crop physiology of black pepper is sparse (Krishnamurthy *et al.*, 2006; Vasantha, 1996 and Varghese-Thomas *et al.*, 1990) and absolutely no literature is available on the physiological or biochemical changes taking place in other Piper species during water stress.

Drought stress is the most common adverse environmental condition that can seriously reduce crop productivity. Increasing crop tolerance to drought stress would be the most economical approach to improve productivity and to reduce agricultural

use of fresh water resource. To survive against the stress, plants have evolved a number of morphological, physiological, biochemical and metabolic responses. Many changes in plant due to drought induced gene expression have been revealed and a large number of genes have been identified. Proteins encoded by some of these identified genes have been confirmed to tolerate drought stress and protect cellular structure or involve in the signal transduction pathway.

There are several production constraints faced by farmers seeking to achieve sustainable yield. Global climate change, inadequate water availability, labour scarcity, coupled with biotic stresses such as epidemic diseases and pests, are the important challenges to be addressed by scientists. In addition to these, consumers expect clean and high-quality spices. In this context, the goal of improvement of black pepper should be for bold berries with high quality and research should also be oriented to producing crop with lower levels of pesticide residues, contamination with adulterants and mycotoxins. The genetic resources of this crop in India are a great strength. International markets receive pepper from Vietnam, Brazil, Indonesia, India, Malaysia, SriLanka. India contributes around 10% to the world market. The productivity of black pepper is slowly declining, mainly because of the prevalence of epidemic diseases, pests and drought.

Pepper suffers due to soil moisture shortage during summer months. Evaluating *Piper* species to water stress gives some knowledge on the stress coping ability of species and if found tolerant, can be used for inducing tolerance in *Piper nigrum* either through conventional or through biotechnological approach. Many physiological and biochemical parameters are used to evaluate black pepper for drought tolerance. These include relative water content, cell membrane leakage, catalase, peroxidase and super oxide dismutase activities (Krishnamurthy *et al.*, 2000). Activities of all these enzymes have been shown to increase during water stress in coconut and the tolerant varieties maintained higher activities than the

susceptible ones (Chempakam *et al.*, 1993). The extent of oxidative stress in a cell is determined by the amounts of superoxide, H_2O_2 and hydroxyl radicals. Therefore, the balance of SOD, ascorbate peroxidase and catalase activities will be crucial for suppressing toxic reactive oxygen species levels in a cell (Apel and Hirt, 2004). It has been shown that membrane leakage and relative water content can be used to screen black pepper germplasm for drought tolerance (Krishnamurthy *et al.*, 2000).

The objective of the present experiment was to investigate physiological and molecular changes in Piper genotypes when subjected to water stress and probability of utilizing some of these parameters to distinguish between susceptible and tolerant types. Tolerance trait if found in any of these species can be transferred to high yielding *P. nigrum* types either through conventional or through molecular approaches.

Review of Literature

2. REVIEW OF LITERATURE

Black pepper (*Piper nigrum L.*) commonly known as king of spices belongs to Piperaceae. Cultivated *Piper nigrum* is predominantly a self pollinated, monoecious crop and it is a tetraploid species having chromosome number 2n=52 (X=13) (Jose and Sharma, 1984). India was the largest black pepper exporter in the world with an area of 1, 80,000 ha and production of 43,000 tonnes (Spices Board, 2012). Major constraints for reduced production of pepper are traditional cultivars with poor genetic potential, drought, poor management, foot rot disease caused by *Phytophthora capsici*. In Kerala water deficit during off season i.e., from December-May is common where black pepper is mainly grown. The literature was abundant on drought in plantation crops but on black pepper, research is scanty. (Vasantha and Ramadasan, 1990).

2.1. Black pepper varieties used in the study

PRS 44 (Panniyur- 1) is the first hybrid variety developed at Pepper research station, Panniyur, 1996. It is the F1 selection of Uthirankotta X Cheriyakaniyakadan with an average yield of 1242.0 (Kg/ha). Panniyur-1 is suitable to all pepper growing regions, adopted to shade areas with long spikes, bold berries and having high oleoresin content. PRS 115 (Panniyur-5) also developed at Pepper research station, Panniyur during 1996. It is an open pollinated progeny of Perumkodi with an average yield of 1107.0 (Kg/ha). It is a shade tolerant, long spiked, with moderately high oleoresin content and suited for mixed cropping in gardens (Spices Board, 2012). Karimunda is a bisexual cultivar with high yield and medium quality. Its fresh average yield is 2-3 kg/vine. Spikes are medium in length with medium sized berries and high spiking intensity. It is suitable for intercropping and high density monocropping, PRS-153 (Panniyur-8) released from PRS, Panniyur during the year 2013. Hybrid of Panniyur 6 X Panniyur 5 were reported as drought tolerant with a reported yield of 2000 kg/ ha. PRS-64 (Angamaly), PRS-156 (*Piper attenuatum*),

PRS-160, PRS-155 (IPC-32), PRS-149 (N.E. Fragrance), PRS- 161 are the cultivars developed at Pepper research station, Panniyur. *Piper attenuatum* used in ayurvedic medicine and it is a rare species. PRS-160 is an open pollinated progeny of 'Balankota' with an average yield of 2570 kg/ha. To maintain the yields in black pepper, planting drought tolerant varieties is one of the methods to mitigate water stress (Thankamani and Babu, 2014).

2.2. Drought impact on crop production

Drought is one of the major inherent abiotic constraints that affect agricultural productivity. Drought stress can potentially reduce nearly 20% of crop yield around the world (Scheiermeier, 2008). Plant requires water from seed germination to plant maturation (Athar and Ashraf, 2005) and any degree of imbalance in the uptake of water would cause a serious threat to agriculture by adversely affecting the yield (Wang *et al.*, 2001).

Moisture deficit from March to May is a major constraint in limiting productivity of black pepper in India (Vasantha, 1996). Moisture deficit, affects the establishment of cuttings in the field and photosynthetic ability. Cultivation of drought tolerant variety is one of the solutions to avoid decline in yield during water stress (Rajagopal and Balasimha, 1994).

Under drought stress conditions, 60 per cent reduction in seed yield of green gram genotypes (Naidu *et al.*, 2001). Number of pod production per plant was significantly reduced, due to water stress in French bean (Shivadhara and Singh, 1995). Upreti *et al.* (2000) studied the response of pea cultivars to water stress. The results revealed that pea cultivars differed widely in vigor under the conditions of moisture stress. The effect of moisture stress was more pronounced at vegetative stage than a flowering stage.

Thakur *et al.* (2000) reported that due to water deficit conditions there was reduction in the plant height and maximum reduction was observed under 75 per cent water deficit (6.0 cm) compared to control (9.1 cm) in chilli. Luz *et al.* (1997) reported that water stress reduced cotton yield with greatest effect at the flowering and fruiting stages.

In 2004, the drought is estimated to have affected 2 million ha of cropped area and over 8 million people in Thailand (Bank of Thailand 2005 and Asia Times 2005). In 2004, a severe drought hit many countries in South-east Asia and caused the shriveling of crops on millions of hectares, shortages of water, and the suffering of millions of people.

Severe droughts can result in starvation and death of the affected population. Different types of economic costs arise before such severe consequences occur. As a result of market failures, to reduce the negative impact in drought years, farmers attempt to 'self-insure' by making costly adjustments in their production practices and adopting conservative measures.

Understanding the biochemical and molecular responses to drought is essential to unravel plant resistance mechanisms to water stress conditions. Drought impacts include growth, yield, membrane integrity, pigment content, osmotic adjustment water relations, and photosynthetic activity (Benjamin and Nielsen, 2006; Praba *et al.*, 2009). The susceptibility of plants to drought stress varies independence of stress degree, different accompanying stress factors, plant species and their developmental stages (Demirevska *et al.*, 2009). Acclimation of plants to water deficit is the result of different events, which lead to adaptive changes in plant growth and physio-biochemical processes, such as changes in plant structure, growth rate, tissue osmotic potential and antioxidant defenses (Duan *et al.*, 2007). It has become imperative to elucidate the responses and adaptation of crops to water deficit and take

actions to improve the drought resistance ability of crop plants and to ensure higher crop yields against unfavorable environmental stresses.

2.3. Physiological responses:

Many physiological and biochemical parameters are used to evaluate black pepper plant for drought tolerance. These include relative water content, cell membrane leakage, catalase, peroxidase and super oxide dismutase activities. Relative water content and membrane leakage can be used to screen germplasm for drought tolerance in black pepper (Krishnamurthy *et al.*, 2000).

2.3.1. Water relations:

Relative water content is considered a measure of plant water status, reflecting the metabolic activity in tissues. It is used as an index for dehydration tolerance. RWC of leaves is higher in the initial stages of leaf and reduces as the dry matter accumulates. A decrease in the RWC in response to drought stress has been noted in wide variety of plants as reported by Nayyar and Gupta (2006) that when leaves are subjected to drought, leaves exhibit large reductions in RWC and water potential. When plants are subjected to drought stress substantially decreased the leaf water potential, RWC and transpiration rate, with a concomitant increase in leaf temperature (Siddique *et al.*, 2001).

Moisture stress was imposed by withholding irrigation. One set of irrigated plants were also maintained which served as control. All plants were of uniform age at the time of stress induction. The stress treatment continued till the plants started showing wilting symptoms. Before induction of stress, plants were irrigated to field capacity daily (Krishnamurthy *et al.*, 2006).

The two poplar species were submitted to drought stress, the decrease of RWC in the cuttings was 23.3% in *Populus cathayana*, whereas it was 16% in

Populus kangdingensis. The interaction of severity, duration of the drought event and species were affects the RWC (Yang and Miao, 2010).

Study was conducted on RWC and membrane stability of black pepper cultivars from January 1997 to March 1997. Water stress was imposed on rooted black pepper cuttings. Among the 44 varieties/cultivars grown, only 6 varieties viz., Kalluvally 4, Karimunda, Padarpan, Panniyur-5, Poonjarmunda and uthirankotta 2 survived after 4 days of water stress. These varieties showed RWC and membrane stability under water stress. (Thankamani and Ashokan, 2004).

The electrolyte leakage technique to evaluate the cell membrane stability as a mechanism of water stress tolerance in durum wheat and this method is used based on *in vitro* desiccation of leaf tissues by a solution of polyethylene glycol (PEG) (Lutts *et al.*, 2001). It consists of 3 successive steps: (1) a washing treatment to remove solutes from both leaf surfaces; (2) the leaf tissues are plunged in a PEG-solution and (3) a rehydration period during effects of the stress are evaluated.

The components of plant water relations are affected by water stress, stomatal opening and closing is more strongly affected. Moreover, change in leaf temperature is an important factor in controlling leaf water status under water stress. Drought tolerant species maintain water-use efficiency (WUE) by reducing the water loss.

2.3.2. Osmolyte accumulation:

Accumulation of organic and inorganic solutes in the cytoplasm to lower osmotic potential thereby maintaining cell turgor in plants (Rhodes and Samaras, 1994). The maintenance of leaf turgor under drought may also be achieved by the way of osmotic adjustment in response to the accumulation of proline, sucrose, soluble carbohydrates, glycine betaine, and other solutes in cytoplasm improving water uptake from drying soil. The process of accumulation of such solutes water stress is known as osmotic adjustment. Proline is the most widely studied because of

its considerable importance in the stress tolerance. Mobilization of proline was observed to enhance tolerance to water stress in wheat (Nayyar and Walia, 2003).

In order to reduce injury of cells under water stress conditions, accumulation of proline is the immediate response of plants. Accumulation of proline in water stressed maize plants. Proline content increase as the drought stress progressed and reached a peak as recorded after 10 days stress, and then decreased under severe water stress (Anjum *et al.*, 2011b).

Under stress in many plant species, accumulation of proline has been correlated with stress tolerance, and its concentration has been shown to be generally higher in stress-tolerant than in stress-sensitive plants. Under dehydration stress it influences protein solvation and preserves the quaternary structure of complex proteins, maintains membrane integrity and reduces oxidation of lipid membranes (Demiral and Turkan, 2004). Proline can act as a signaling molecule to modulate mitochondrial functions, influence cell proliferation or cell death and trigger specific gene expression, which can be essential for plant recovery from stress (Szabados and Savoure, 2009). Furthermore, it also contributes to stabilizing sub-cellular structures, scavenging free radicals, and buffering cellular redox potential under stress conditions (Ashraf and Foolad, 2007).

2.4. Biochemial responses:

2.4. 1. Reactive oxygen species (ROS):

The ROS generation is one of the earliest biochemical responses of eukaryotic cells to biotic and abiotic stresses. It acts as a secondary messenger to trigger subsequent defense reaction in plants. The production of ROS in plants, known as the oxidative burst, is an early event of plant defense response to water-stress. ROS, which include oxygen ions, free radicals and peroxides, form as a natural byproduct of the normal metabolism of oxygen and have important role in cell signaling. ROS

levels increase dramatically during abiotic stress such as drought resulting in oxidative damage to proteins, DNA and lipids (Apel and Hirt, 2004). Drought induces oxidative stress in plants by generation of reactive oxygen species (ROS) (Farooq *et al.*, 2009). The ROS such as O2, H_2O_2 and -OH radicals, can directly attack membrane lipids and increase lipid peroxidation (Mittler, 2002).

Membrane damage, loss of membrane integrity, and interaction of membrane components with highly reactive oxidation products cause membrane damage and loss of membrane integrity results in membrane ion leakage and accelerated water loss peppers (Kissinger *et al.*, 2006).

Malondialdehyde (MDA) content increases due to drought-induced overproduction of ROS increases and the content of MDA has been considered an indicator of oxidative damage (Moller *et al.*, 2007). It is considered as a suitable marker for membrane lipid peroxidation. The extent of lipid peroxidation caused by ROS reflects reduced membrane stability. The lipid peroxidation is an indicator of the prevalence of free radical reaction. In photorespiration, O_2 uptake loading on the tissues as both processes generate reactive oxygen species, particularly H_2O_2 that produced at very high rates by the glycollate oxidase reaction in the peroxisomes. The increments of the MDA and H_2O_2 concentrations in the water-stressed cuttings were 88.9 and 99.7% in *P. cathayana*, whereas they were only 44and 63.6% in *P. kangdingensis* (Yang and Miao, 2010).

In pea plants, in leaves levels of lipid peroxidation increased two to four fold with an increase in drought stress, and this was highly correlated with protein peroxidation (Moran *et al.*, 1994). Being highly reactive, ROS can seriously damage plants by increasing lipid peroxidation, protein degradation, DNA fragmentation and ultimately cell death.

2.4.2. Role of antioxidant enzymes:

Plants have an internal protective enzyme catalyzed clean up system to avoid injuries of active oxygen, thus guaranteeing normal cellular function (Horvath *et al.*, 2007). The activities of antioxidative enzyme and ROS production, determines whether oxidative signaling and damage will occur (Moller *et al.*, 2007). Plants have evolved a complex enzymatic and non-enzymatic antioxidant system to minimize the effects of oxidative stress, such as low-molecular mass antioxidants (glutathione, ascorbate, carotenoids) and ROS scavenging enzymes superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX) (Apel and Hirt, 2004). Under oxidative stress non-enzymatic antioxidants cooperate to maintain the integrity of the photosynthetic membranes.

Scavenge ROS directly by the enzymatic components or may act by producing a non-enzymatic antioxidant. Yang *et al.* (2009) compared with 100% field capacity, at 25% field capacity the increased activities of CAT, SOD, POD, APX and GR were 4.3, 103, 172, 208 and 56% in *P. cathayana*, respectively, whereas they were 8.1, 125, 326, 276 and 78% in *P. kangdingensis*.

Increase in enzyme activities of peroxidase and SOD were observed in tolerant black pepper varieties by inducing soil moisture stress. In Panniyur-1 high levels of lipid peroxidation were observed, which drought susceptible variety is. The best biochemical characters were observed in Panniyur-5 during water stress. In this adaptation Panniyur-1 was relatively very poor. (Thankamani *et al.*, 2002)

The concerted action of antioxidants causes destruction of O_2 and H_2O_2 in plant cells. In the chloroplast, mitochondrion, cytoplasm and peroxisome, the O_2 can be dismutated into H_2O_2 by SOD. Scavenging H_2O_2 by POD which was produces through dismutation of O_2 catalyzed by SOD. CAT is a main enzyme and found in peroxisomes to eliminate in the mitochondrion and micro body (Shigeoka *et al.*,

2002) and thus help in ameliorating the detrimental effects of oxidative stress, but considered indispensable for decomposing H_2O_2 during stress. The higher level of antioxidative enzyme activities may contribute to drought induction by increasing the capacity against oxidative damage (Sharma and Dubey, 2005). Scavenging ROS by antioxidant enzymes and reduce the damaging effects may correlate with the drought resistance of plants.

In coconut the activities of all these enzymes have been shown to increase during water stress and the tolerant varieties maintained higher enzyme activities than the susceptible ones (Chempakam *et al.,* 1998). The oxidative stress in a cell is determined by the quantity of superoxide, H_2O_2 and hydroxyl radicals. Hence, the balance of SOD, ascorbate peroxidase and catalase activities will be crucial for suppressing toxic reactive oxygen species levels in a cell (Apel and Hirt, 2004).

2.5. Molecular basis of drought tolerance

During water-stress conditions the induced genes are thought to function in protecting cells water deficit by the production of important metabolic proteins and regulation of genes for signal transduction in water stress response. A number of droughts responsive genes were cloned and characterized from different plant species (Nepomuceno et al., 2000). Transcription of many of these genes is unregulated by drought stress. Initial attempts to develop transgenics (mainly tobacco) for abiotic stress tolerance. It involves 'single action genes' that would confer increased tolerance to abiotic stresses. Stress-induced proteins with known functions such as water channel proteins, key enzymes for osmolyte (proline, betaine, sugars such as trehlose, and polyamines) biosynthesis, detoxification enzymes, and transport proteins were the initial targets of plant transformation.

In several species, various genes respond to drought stress and functions of their gene products have been predicted from sequence homology. Hence, such genes

provides tolerance to the plant, that are induced during drought stress conditions by functions in protecting cells from water stress by the production of important metabolic proteins and regulating other genes for signal transduction (Shinozaki and Yamaguchi-Shinozaki, 1996). Three major groups of the gene products: (1) the genes that encode products and protect plant cells against stresses. E.g. Heat stress proteins or chaperones, LEA proteins, osmoprotectants, antifreeze proteins, detoxification enzymes and free radicals scavengers (Bray *et al.*, 2000). (2) The genes that are involved in signaling cascades and in transcriptional control. E.g. MAPKs, CDPKs (Ludwig *et al.*, 2004) and kinase (Zhu, 2001), phospho lipases transcriptional factors (Shinozaki and Yamaguchi-Shinozaki, 2000). (3) The genes that are involved in water and ion uptake and transport such as aquaporins and ion transporters.

2.5.1. Genes and proteins associated with drought

2.5.1.1. The role of Heat shock proteins and LEA-type proteins

During environmental stress, plants activate a set of genes leads to the accumulation of specific stress associated proteins (Vierling *et al.,* 1991). Heat shock proteins and late embryogenesis abundant proteins are two major types of stress induced proteins. They have been shown to play a major role during the stress in cellular protection (Vierling and Kimpel, 1992). Hsps have been shown to act as molecular chaperones, which are responsible for protein synthesis, targeting, maturation and degradation in a broad array of normal cellular processes. Under stress conditions, molecular chaperones function in the stabilization of proteins and membranes, and in assisting protein refolding.

Among the five conserved Hsps families (Hsp100, Hsp90, Hsp70, Hsp60 and sHsp), the small heat shock proteins (sHsps) are most prevalent in plants (Vierling, 1991). sHsps are not only expressed in response to heat shock but also under water, salt, and oxidative stress, and at low temperature (Hamilton and Heckathorn, 2001).

However, some members of the plant sHsps can also stabilize or reactivate inactivated enzymes (Lee *et al.,* 1995). During water stress, heat shock proteins like hsp 27 and hsp 70 also be induced in soyabean and maize respectively (Sachs and David, 1986). Over expression of late embryogenesis abundant proteins was correlated with desiccation tolerance (Villalobos *et al.,* 2004). Over expression of LEA protein (HAV1,) from barley conferring dehydration tolerance to transgenic plants (Chandrababu *et al.,* 2001).

2.5.1.2. Role of Compatible osmolytes:

"Compatible osmolytes" are the class of small molecules includes certain amino acids (proline), quaternary ammonium compounds (e.g. glycinebetaine, prolinebetaine, B-alaninebetaine, and choline-O-sulfate) and the tertiary sulfonium compound 3-dimethyl sulfonio propionate (DMSP) and these are derived from amino acid precursors. During stress condition osmolytes are involed in signaling/regulating plant responses, including reduced growth against stress. The common osmolytes are proline, trehalose, fructan, mannitol and glycinebetaine. Transgenic plants overproducing osmolytes often exhibit impaired growth.

Fructose-1,6 bisphosphate (FBP) aldolase catalyzing the reversible aldol condensation/cleavage reaction between glyceraldehydes 3-phosphate and dihydroxy acetone phosphate to yield FBP. It is known as a key enzyme in cellular metabolism, which is involved in multiple pathways including glycolysis, gluconeogenesis and the calvin cycle. FBP aldolases are distinguished into two classes based on their structure and mechanistic properties (Perham, 1990). Several isoenzymes of FBP aldolases exist in higher plants which are localized to the cytoplasm or the chloroplast or the chloroplast (Razdan *et al.,* 1992). The enzyme FBP aldolases are involved in the glycolytic pathway in cytoplasm, whereas the enzyme is part of the Calvin cycle in the chloroplast. The regulatory role of this enzyme has highlighted since last two decades. The moderate decrease of plastid aldolase activity inhibits alters the levels

of sugars, starch, inhibits photosynthesis and growth of potato plants (Heak *et al.*, 1998). Chloroplastic enzyme gene is differentially expressed under temperature stress (Michelis and Gepstein, 2000), salt-stress (Zorb *et al.*, 2004; Nguyen et al., 2006), herbivorous attack (Voelckel and Baldwin, 2003), and in selenium metabolism (Agalou *et al.*, 2006). Understanding the mechanism of drought tolerance in plants, the evidences of over expression of this gene under biotic and abiotic stresses make it a strong candidate for study under drought stress.

The common metabolic responses of higher plants to water deficits, salinity stress are Proline accumulation (Rhodes *et al.*, 1999). It protects membranes and proteins from the adverse effects of high concentrations of inorganic ions and temperature extremes (Santoro *et al.*, 1992). It is also function as a protein-compatible hydrotrope (Srinivas and Balasubramanian, 1995) and as a hydroxyl radical scavenger (Smirnoff and Cumbes, 1989).

Accumulation of greater quantities of proline in hydroxyl proline-resistant mutants of barley and winter wheat than in wild-type (Droffling *et al.*, 1993). But, the concentrations of proline accumulated by these mutants may be an order of magnitude smaller than required to produce a significant physiological effect on osmotic stress tolerance. The hydroxyl proline-resistant lines are significantly more frost tolerant in winter wheat than wild-type (Droffling *et al.*, 1993). Proline accumulation in maize root apical meristems in response to water deficits involve increased proline deposition to the growing and appears to require abscisic acid (ABA) (Sharp *et al.*, 1994).

In many microorganisms, trehlose protects biological molecules in response to stress conditions. It is a non-reducing disaccharide, made from UDP-glucose and glucose-6-phosphate involves two steps. The first step is conversation of UDP-glucose and glucose 6-phosphate to trehlose -6-phosphate is by trehlose-6- phosphate synthase, encoded by an otsA, the bacterial gene. In second step, glucose-6-

phosphate to trehlose by a phosphate encoded by the otsB gene. Tobacco plants with bacterial otsA have a greater ability to photosynthesize under water stress.

2.5.1.3. Vitamin C

Ascorbic acid (Asc) is the most abundant antioxidant in plants and it is present in milli molar concentrations that range from 10 to 300mM (Smirnoff, 2000). It serves as the major contributor to the cellular redox state and It is used by ascorbate peroxidase (APX) to convert H_2O_2 to water.

Stomatal movement is also controlled by the divergent pathway ofH_2O_2 (Kohler *et al.*, 2003). Ascorbate is consistent with its role as a scavenger of $H_2O_2$and the H_2O_2 induced stomatal closure was reversed by exogenous application of ascorbate (Zhang *et al.*, 2001). Consequently, plants with to exhibit reduced responsiveness to ABA or H_2O_2 signaling are predicted due to increased ascorbate.

The crop drought tolerance was increases by decreasing the amount of an enzyme that is responsible for recycling vitamin C, which is the development of technology reported by the researchers from University of California (Chen and Gallie, 2004).The decreasing amount of the enzyme dehydro ascorbate reductase or DHAR would reduce the ability of plants to recycle vitamin C, making them more drought tolerant through improved water conservation. The plants own gene was used by the researches to decrease the amount of the enzyme three fold. Tobacco is used as a model for crops that are level of vitamin C could be boosted by increasing the amount of this same enzyme.

As vitamin C is an important antioxidant in plants and among its many functions in both, it destroys ROS and damage or even kills cells. Vitamin C must be regenerated if once used, otherwise it is irrevocably lost. The enzyme DHAR plays a critical role in this recycling process. The reduction in recycling causes plants, highly responsive to dry growth conditions by reducing the rate of water that escapes from

their leaves. Thus, the plants are able to grow with less water and survive in a drought.

2.5.1.4. Polyamines in stress tolerance

Polyamines have been involved in many physiological processes such as growth, development and a role for these growth regulators in stress response of plants has been proposed. Polyamines are organic compounds having two or more primary amino groups that are growth factors synthesized in cells via highly-regulated pathways in eukaryotic and prokaryotic organisms. Polyamines such as spermidine, spermine, putrescine and cadaverine. The enzymes ornithine decarboxylase and arginine decarboxylase controls biosynthesis of polyamines in plant, which are required for production of putrescine and S-adenosyl- L- methionine (SAM) decarboxylase that is necessary for the formation of spermidine and spermine. Experimental evidences support that polyamines role in stress tolerance of plants (Urano *et al.*, 2004). Although a function as ROS scavengers was initially proposed (Tiburcio *et al.*, 1994). The interaction of the compounds of growth regulators with bent adenine tracts in double-stranded DNA suggests that direct role in differential gene transcriptional activity.

2.5.1.5. Abscicic acid (ABA) responsive genes

Various environmental stresses may trigger the similar transduction pathways. ABA (abscicic acid) is a plant hormone which accumulates in response to drought or other stresses and reduction in endogenous ABA content is to induce certain water deficit induced genes. During water deficit, accumulation of ABA is one of the steps in signal transduction pathways that induce genes. Different protein kinases have been reported and that are function in phosphorylation in various signal transduction pathways, including ABA responses and water stress.

Under water stress in the 8 days old cowpea, 9-cis-epoxycarotenoid dioxygenase gene (NCED), induced by ABA synthesis (Luchi *et al.,* 2000).

The stress induced responses due to ABA mediated or independent of ABA (Shinozaki and Yamaguch-Shinozaki, 1996). Protein synthesis may or may not require for ABA mediated gene response. ABA induced mRNA of rd22 gene which showed homology to an unknown seed protein of *Vicia faba,* required protein synthesis since cycloheximide restricts induction of the gene (Yamaguch-Shinozaki, 1996).

2.5.2. Transcription factors and their significance in plant stress tolerance

Multiple signaling pathways regulates plant stress responses that activate gene transcription and its downstream machinery, contain a large number Transcription factors (TFs) of the plant genomes e.g. *Arabidopsis* genome contains about 5.9% of its coding for more than 15000 TFs (Riechmam *et al.,* 2000).TFs belongs to a large multigene families, e.g. MYB, AP2/EREBP, Bzip and WRKY. However, Individual members of the same family respond differently to different stress stimuli; but some stress responsive genes may share the similar TFs, as indicated by the significant overlap of the gene-expression profiles (Kreps *et al;* 2002).

The C-repeat binding factors (CBF) and dehydration-responsive transcription factors (DREB) bind to CRT cis-acting and DRE elements with same motif (CCGAC). Stress inducible members of the CBF/DREB1 family, such as CBF 1, CBF 2, and CBF3. are by AP2/ EREBP families encodes DREB/CBF proteins and mediate the transcription of genes such as rd29A,rd17, cor6.6, cor15a, erd10, kin1, kin2 and others in response to water and cold stress (Thomasshow *et al.,* 2001).

In transgenic *Arabidopsis*, an active form of DREB2 was trans activate target stress-inducible genes and it tends to improve drought tolerance (Sakumam *et al.,* 2006). No Apical Meristem (NAM) are the plant specific transcription factors and

commonly known as NAC proteins. The NAC protein, ANAC072/*RD26*, may act as a transcription factor in response to drought and ABA stress. Over expression of this gene results in hyper sensitivity to ABA and up regulation of a large range of stress inducible genes (Fujita *et al.*, 2004).

TFs also act as repressors of the water stress. The AP2 domain TF, ABA repressor 1 (ABR1) is up-regulated by drought stress. Under stress conditions, the stress sensitive *abr* 1 null mutant has increased expression of stress response genes (Pandey *et al.*, 2005). AP2 transcription factor ethylene ERF7 is the negative regulator of ABA response. Over expression of ERF7 results in drought sensitivity and increased stomatal aperture (song *et al.*, 2005).

ABA response element binding proteins (*AREB/ABF*), a sub-family of bZIP TFs are well characterized group of TFs involve in the water stress. *ABF* TFs expressions is induced by ABA treatment. *ABF1* is induced by cold stress, *ABF2 (AREB)* and *ABF3* are induced by salt stress and *ABF4* is also induced by drought, cold stress and salt stress (Choi *et al.*, 2000). TFs except*ABF1*, have a positive role as growth regulators, seed germination and stomatal control (Kang *et al.*, 2002).

TFs containing zinc fingers are involved in drought stress. *ZAT12* is a zinc finger protein induced by water stress, as well as other stresses (Davletova *et al.*, 2005). Over expression of the protein results in osmotic stress tolerance, either it acts in an ABA-dependent (or)ABA-independent pathway (Davletova *et al.*, 2005).*AZF2 and ZAT10* are the zinc finger TFs is appear to be a negative regulators of the water stress (Sakamoto *et al.*, 2004).

2.5.3. Aquaporins

Aquaporins are membrane spanning proteins, the major intrinsic proteins (MIP's) and have conserved structures. It facilitates the movement of water by creating pores in the lipid bilayer through plasma membrane, thus it increases the rate

of the water flow. These proteins are mostly expressed in rapidly growing tissues and in root cells with an intense water flux. These protein channels can be expressed facultative in response to the extracellular water potential. The aquaporine gene was up-regulated and down-regulated by dehydration and salinity respectively (Smith–Espinzoa *et al.*, 2003).

2.6. Differential display reverse transcription polymerase chain reaction (DDRT-PCR)

Differential display reverse transcription polymerase chain reaction (DDRT-PCR) or differential display of eukaryotic mRNA was first introduced by Liang and parde in 1992. It is used to identify and compare the differentially expressed genes. The disadvantages of methods such as screening differential hybridization and subtractive library are (1) Construct and screen cDNA libraries (2) The requirement for large amounts of RNA (3) Un recognition of subtle changes (4) Study only two cell populations or variables.

Advantages of DD (Differential display) are simple and established methods, it is reproducible and sensitive; it does not require information about proteins, more than two samples can be compared and only a small quantity of starting material is required (Yamazaki and Saito, 2000).

To generate cDNA fragments, anchored and arbitrary primers are used by reverse transcription, followed by PCR (RT-PCR). In sequencing gel the cDNA fragments are resolved and compared which reflects differences in the mRNA composition. Further, it can be eluted, cloned and sequenced.

To obtain intact, DNA free RNA with DD residues then converting the mRNAs from cells into cDNAs, that differs at the last 3' non-T base using 3 individual anchored oligo-dT primers. The beginning of the poly (A) tail for any given mRNA enables the homogeneous initiation of cDNA synthesis. In the presence

of a set of second primers by PCR, cDNAs are further labeled with isotopes that are short and arbitrary sequence. To maximize the number of amplified mRNA, the annealing temperature of the PCR is low. (Liang and parde, 1992). Comparison of the cDNAs runs side-by –side on a denaturing polyacrylamide gel.

Limitations of these methods are the differential expression pattern was not always be reproducible on Northern blots and the little information was gained from sequence. At extreme 3' end of the mRNA, only ~300 bp of cDNAs obtained and the failure to selective reamplify the most of DD cDNA products.

The low stringency annealing, isolation of co-migrating PCR products and that many of one products were reamplified with arbitrary primer are the major reasons for false-positive. The development of one-base anchored primers and rationally designed 13 mer primers have increase the accuracy of DD. reamplification or screening.

Various methods have been derived from DD such as, targeted display, ordered differentially display (restricted endonucleolytic analysis of differentially expressed sequences; READS).

Advanced methods have been introduced that have both advantages and disadvantages over DD. One of the most important methods is microarrays. The microarrays and DD are theoretically simple, but the fundamental difference is that DD visualizes the mRNAs after amplification in subsets directly and labeling in contrast with microarrays where the mRNAs are visualized indirectly after hybridization of complex mRNA probes as first strand cDNAs to a mostly incomplete set of cDNA templates spotted on a glass surface. Microarrays have all gene sequences before a chip can be made is one of the major challenges. For any living organism DD is capable of detecting both known and novel genes.

The genes isolated using DD from plants that occur in small amounts and thus are characteristically difficult to identify and encode membrane proteins and transcription factors. These are involved in signal transduction, stress response and secondary metabolism (Yamazaki and Saito, 2002). This is a powerful technique for isolating cDNA specifically in stress induced cells as well as identifies genes repressed to stress or pathogen invasion. The flexibility of DD is to identify and isolate transcripts, up and down regulated, from multiple samples on a single gel.

2.7. *In silico* analysis

The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between the primary biological sequences, such as the amino-acid sequences of different proteins or the nucleotides of DNA sequences. A BLAST search enables a researcher to compare a query sequence with a library or database of sequences, and identify library sequences that resemble the query sequence above a certain threshold. (Altschul *et al.*,1990). BLAST has family of programs like Blastn, given a DNA query, returns the most similar DNA sequences from the DNA database that the user specifies (Blast NCBI) and Blastx compares the six-frame conceptual translation products of a nucleotide query sequence (both strands) against a protein sequence database. .

Materials and Methods

3. MATERIALS AND METHODS

The study on "Molecular characterization of black pepper (*Piper nigrum* L.) genotypes for drought tolerance" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), IT–BT Complex, College of Horticulture, Vellanikkara during the period from August, 2013 to June 2015. Materials used and methodologies adopted for the study is described in this chapter.

3.1.1. Plant materials

Pepper research station (PRS), Panniyur maintains germplasm accessions of black pepper under field conditions. Screening and evaluation of genotypes were carried out to identify genotypes showing tolerance to water stress, under field conditions. Based on the phenotypic parameters 15 genotypes were identified. These genotypes were characterized using morphological characters at PRS, Panniyur. Among them ten genotypes were taken for the present study to characterize the genotypes for drought tolerance on phenotypic and molecular basis.

Ten black pepper (*Piper nigrum* L.) genotypes PRS-64(Angamaly), PRS-44(Panniyur-1), PRS 155(wild type), PRS-149(wild type), PRS-156(*Piper attenuatum*), PRS 160(Hybrid), PRS 161(Hybrid), PRS 153(Panniyur-8), PRS-115(Panniyur 5) and Karimunda were used in the present study (Plate 1). The rooted cuttings were obtained from the Pepper research station (PRS), Panniyur.

3.1.2. Chemicals, glassware and plastic ware

All the chemicals used in the study were of good quality (AR/GR grade) obtained from Merck, Sisco Research Laboratories, Himedia and Sigma. Molecular grade enzymes, buffers, and DEPC water supplied by Bangalore GeNei Ltd. RNase ZAP from Ambion, Inc, USA. DDRT-PCR kit namely RNA image kit (Gen Hunter-USA).

Plate 1: Maintenance of rooted cuttings of pepper genotypes in pots

3.1.3. Laboratory equipments

Instruments available at CPBMB and Department of Seed Technology were utilized for the work while Bioinformatics related softwares was accessed from Distributed Information Centre (DIC).

3.2 Screening of black pepper genotypes for drought tolerance

3.2.1. Treatment to the plants

The selected genotypes were given two treatments. Plants without any treatment served as control and water was withheld continuously for 15 days served as treated and observations were recorded at 5 days interval on six month old rooted cuttings for physiological analysis. Eight pots were maintained under greenhouse conditions for each variety. Each pot with the size of 80 cm X 80 cm contains potting mixture. The design used was completely randomized block design.

Treatments details

1. Control
2. Water stress

3.2.2. Measurement of biometric parameters

The plant observations were taken on leaf fresh weight, dry weight and number of days taken for wilting for biometric analysis after water is withheld continuously for 15 days and observations were noted at 5 days interval on six month old rooted cuttings. Statistical analysis was carried out using Duncans multiple range test.

3.2.3. Measurement of physiological parameters

The physiological parameters like relative water content, electrical conductivity, Membrane integrity was monitored in all the treatments after water was withheld continuously for 15 days and observations are noted at 5 days interval on six month old rooted cuttings. All these parameters were monitored using EC meter.

Based on the physiological parameters the most drought tolerant genotype will be identified and that will be subjected to molecular analysis.

3.3. Transcriptome analysis of drought tolerant genotype

3.3.1. Total RNA extraction and normalization

A. General precautions for RNA isolation

To obtain intact and high quality RNA, precautions were strictly followed to avoid contamination and degradation during RNA isolation. All the materials like glassware, mortar and pestle, micro tips and micro centrifuge tubes were treated with DEPC treated water and then double autoclaved. Solutions like 80 per cent ethanol and MOPS buffer were prepared with double autoclaved DEPC treated water. Electrophoresis unit was first wiped with 80 per cent ethanol or RNAse ZAP and then washed with DEPC treated water.

B. Sample collection

Young, tender leaves from the two different treatments of most drought tolerant genotype (PRS-64) i.e, Control plants and Water stress plants were collected early in the morning for RNA isolation. Leaves were then washed quickly with DEPC treated water.

C. Isolation of total RNA

Isolation of good high quality and quantity of RNA is a pre requisite for differentially displayed reverse transcriptase PCR analyses (DD-RT-PCR). Total RNA was extracted from the treatments using TRI reagent method (Sigma- Aldrich, USA).

Reagents

TRI reagent (Sigma- Aldrich)

Chloroform

Ice cold Isopropanol

75 per cent ethanol (v/v)

Autoclaved DEPC treated water

Procedure

1. 0.1 g of leaf tissue was weighed and ground well in liquid nitrogen.
2. One ml of Trizol per 100 mg of leaf tissue was added and the homogenate was incubated till the solution became brown.
3. The homogenate was transferred to a 2ml micro centrifuge tube.
4. The contents were centrifuged at 12,000 g at 4^0 C for 10 min.
5. The supernatant was transferred to a 2ml micro centrifuge tube.
6. Two hundred micro liters of chloroform was added to the contents mixed by shaking. It was then kept at room temperature for 10 min.
7. The contents were then centrifuged at 12,000 g at 4^0C for 15 min.
8. The supernatant was transferred to another fresh 1.5 ml micro centrifuge tube.
9. 500 micro liters of isopropanol was added to the supernatant. The contents were mixed by slight inversion then incubated for 10 min and centrifuged at 12,000 g at 4^0C for 10 min.

10. The supernatant was discarded and the pellet was air dried under laminar air flow (LAF) for 10 min.
11. The pellet was dissolved in 30 µl of double autoclaved DEPC water.

3.3.3 Quality and quantity analysis of total RNA

3.3.3.1 Quality analysis by Formaldehyde-agarose gel electrophoresis

The gel (1.2% agarose) electrophoresis was performed to check the quality of RNA, following the method as described by Sambrook *et.al.* (1989).

Materials required for agarose gel electrophoresis

1) Agarose (GeNei, Low EEO)
2) 10 X MOPS buffer (pH 7.0)
3) 6X Loading/ Tracking dye
4) Electrophoresis unit, power pack (GeNei), casting tray, comb
5) Ethidium bromide solution (0.5µg/ml)
6) UV transilluminator (Herolab ^{R})
7) Gel documentation and analysis system (UVP, GelDoc It^{TM} imaging system; UK)

Chemical composition of buffers and RNA loading dye are given in Annexure I.

Procedure

1. Eight hundred ml of 1X MOPS buffer was prepared by diluting 10X stock solution with autoclaved DEPC treated water. This buffer was used to fill the electrophoresis tank and to prepare the gel.
2. The open ends of gel casting tray were sealed with cello tape and placed on a horizontal platform. The comb was placed at one end properly.

3. 1.2 percent agarose was dissolved properly in 1X MOPS buffer by boiling in a microwave oven.
4. The solution was poured into the gel casting tray and allowed to solidify.
5. After the gel was completely set (20 to 30 min. at room temperature), the comb and cello tape were carefully removed.
6. The gel was placed in the electrophoresis tank containing 1X MOPS buffer (with the wells near the cathode) such that it is submerged to a depth of 1 cm.
7. One micro liter of 6X gel loading dye was mixed with 2µl (20 µg/lane) of RNA and then loaded in the wells. λ DNA / ECORI + HINDIII double digest (Bangalore GeNei) marker was loaded in the first well.
8. The cathode and anode were connected to the power pack and the gel was run at a constant voltage of 25 volts.
9. The power was turned off when the tracking dye moved 2cm from the wells.
10. The gel was placed in a gel documentation system and the bands were visualized under UV light and the gel image documented.

3.3.3.2 Spectrophotometric analysis of RNA

The samples which gave three discrete bands specific to RNA on an agarose gel electrophoresis was further analyzed. The quantity and quality of RNA was estimated using Nano Drop® ND-1000 spectrophotometer (Nano Drop Technologies Inc., USA). Before taking samples readings, the instrument was set to zero by taking 1 µl double autoclaved DEPC treated distilled water as blank. One micro liter of RNA from each sample was used for quantification. The absorbance of nucleic acid samples were measured at a wavelength of 230/280, OD_{260}/OD_{280} ratios was recorded to assess the purity of RNA.

A ratio of 1.8 to 2.0 for OD_{260}/OD_{280} indicated good quality RNA. Good quality total RNA from all two treatments was pooled separately.

3.3.4 Differential Display Reverse Transcription Polymerase Chain Reaction (DD-RT-PCR)

Differential display reverse transcription polymerase chain reaction (DD-RT-PCR) was first introduced by Liang and Parde in 1992, to identify and compare differentially expressed genes. The DD technique was developed with the aim of overcoming limitations of methodologies previously used for identifying differentially expressed genes.

DD has advantages over these techniques because it is based on simple and established methods, it is reproducible and sensitive, it does not require biochemical information about proteins, more than two samples can be compared simultaneously, and only a small amount of starting material is needed.

The method is based on using sets of anchored and arbitrary primers to generate cDNA fragments by reverse transcription, followed by PCR (RT-PCR). The cDNA fragments are resolved and compared in sequencing gels. The resulting cDNA patterns reflect differences in the mRNA composition. DD cDNAs can be isolated, cloned and sequenced.

3.3.4.1 First strand cDNA synthesis

The isolated total RNA predominantly consists of rRNA, tRNA and mRNA. The isolation of intact and good mRNA is very essential for successful first strand synthesis and subsequent PCR amplication. Mature mRNA molecules carry poly (A) at their 3' termini, to which oligo dT primers anneal. Reverse Transcription enzyme can synthesize cDNA from isolated RNA under favorable conditions. The single stranded cDNA from isolated RNA under favorable conditions. The single stranded cDNA was used immediately for PCR without further purification.

Materials

1. RNA sample
2. Oligo $(dT)_{18}$ primer(500µg/ml)
3. First strand buffer (5X)
4. 10mM dNTP mix (2.5 mM each)
5. DTT(0.1 M)
6. M-MuLV reverse transcriptase (200 units/µl)
7. RNase OUT Recombinant Ribonuclease Inhibitor (40 units/µl)
8. Autoclaved DEPC treated water

Procedure

1. 3µl of total RNA (1-5µg) was added to a 0.2 ml micro centrifuge tube.
2. Added 1 µl of oligo dT primer.
3. Added 1 µl of 10mM dNTP mix.
4. Added 7 µl of sterile distilled water.
5. The reaction mix was incubated at 65^0C for 5 min in a thermo cycler and then quick chilled on ice.
6. The reaction mix was spinned briefly to collect the content at the bottom of the tube and the reagents were added one by one as follows.
    - 1 µl RNase OUT Recombinant Ribonuclease Inhibitor (40 units/ µl)
    - 2 µl DTT
    - 4 µl First strand buffer (5X)
7. The content was mixed gently and incubated at 37^0C for 2 min.
8. Added 1 µl M-MuLV reverse transcriptase and mixed by pipetting gently up and down.
9. The content was incubated at 37^0C for 1 hour and 30 min.
10. Inactivated the reaction by heating at 70^0C for 15 min.

3.3.4.2 Second strand c DNA synthesis

Each fraction of the single stranded cDNA was amplified by PCR with the anchored and respective arbitory primers.

Table1. List of Anchor (T11C) and Arbitrary primers (AP) used in DD-RT-PCR

Primer No.	Sequential information(5' to 3')	No. of bases
T11C	AAGCTTTTTTTTTTC	16
AP1	AAGCTTGATTGCC	13
AP2	AAGCTTCGACTGT	13
AP3	AAGCTTTGGTCAG	13
AP4	AAGCTTCTCAACG	13
AP5	AAGCTTATAGGC	13
AP6	AAGCTTGCACCAT	13
AP7	AAGCTTAACGAGG	13
AP8	AAGCTTTTACCGC	13

Procedure

Master mix prepared as indicated in the table 2 below and spun briefly for proper mixing. Later, aliquot of the master mix was added to each tube and cDNA template was added separately.

1. A negative control was maintained to ensure that no non-specific amplification has occurred due to contamination in any of the reagents.
2. Special thin walled tubes of uniform thickness were used to ensure rapid and equal temperature changes throughout the reaction volume.
3. PCR reaction was standardized for second strand synthesis as detailed in table 2.

Table2. Composition of reaction mixture for PCR

Components	Volume per reaction (µl)
10X reaction buffer A	2.0
dNTP mix (25 µM)	1.6
T11C Anchored primer (2 µM)	2.0
AP(1-4) Arbitrary primer(2 µM)	2.0
cDNA	2.0
Taq DNA polymerase	0.2
Nuclease free water	10.2
Total	20.0

Table 3. Temperature profile for second strand cDNA synthesis

SI No.	Step	Temperature	Time
1	Initial denaturation	94^0C	2min
2	Denaturation	94^0C	30s
3	Annealing	40^0C	2min
4	Extension	72^0C	1min
5	Step 2 to 4	40 cycles	
6	Final extension	72^0C	10min
7	Hold	4^0C	10min

PCR product was stored at -20^0C, later electrophoresed using 6.0% Poly Acrylamide Gel Electrophoresis (PAGE) and visualized by silver staining.

3.3.4.3 Urea Poly Acrylamide Gel Electrophoresis of DD-RT-PCR fragments

The cDNA or transcript derived fragments from the two different treatments were visualized in 6% denaturing poly acrylamide gel electrophoresis by silver staining (Liang and Parade, 1992).

a. Plate preparation

One large (180 x 250 cm) and one smaller (180 x 225 cm) glass plate were carefully cleaned and rinsed with water and subsequently with 70% ethanol. Particular attention was paid to the inside surface of the plate. Large plate i.e., Repel plate coated with 600 µl of repel saline and small plate with 10µl of bind saline (methacryloxypropyl trimethoxy saline) dissolved in autoclaved 990ml distilled water to prevent the gel from sticking to the repel plate. Spacers (0.25 mm) were placed along the sides of the larger plate. The edges of the two plates were aligned and the plates were clamped together with clamps.

b. Gel preparation

Plates were placed nearly horizontal and the comb inserted. The gel solution (final concentration of 6%) was prepared by mixing 7.5 ml of 38 % acryl amide/ 2% N ,N' –methylene bis- acryl amide (19:1), 21 g urea, 5 ml 10X TBE and autoclaved distilled water to final volume of 50 ml. 50 µl of TEMED (N,N,N',N'- tetra methyl ethylene diamine) and 1ml of 10 % (w/v) ammonium per sulfate solution (APS) was added to the 50 ml acryl amide solution. The acryl amide gel solution was gently mixed and poured between the plates at an angle of 45^0C avoiding any trapped air bubbles. When the plates were almost full, it was placed nearly horizontal and the comb inserted. The gel was allowed to polymerize for 20 min. The glass plates were assembled into the electrophoresis system. The upper buffer chamber and the bottom chamber were filled with 0.5X TBE buffer. Pre run was given at 1500 Volt for 45 min after pre run the power was switched off.

3.3.3.4 Sample preparation and electrophoresis

Samples were prepared by mixing 4 µl of sample with 2 µl Loading dye (10 µl formamide, 0.5M EDTA, pH 8, 10mg of xylene cyanol and 10 mg of bromo phenol blue) Samples were then loaded into the well. Electrophoresis was conducted at 1200 volt for approximately 3 hr. After the xylene cyanol reached 2/3 portion of the gel, the electrophoresis was terminated.

3.3.4.7 Silver staining of the gel

After completion of electrophoresis, the two glass plates were carefully separated from each other. The bind plate with the gel was fixed in 8% acetic acid for 20 min and then washed in distilled water for 7 min, washing was performed for four times then gel was kept in 0.1% silver nitrate solution for 20 min for staining. It was then washed in distilled water for 5 min and then transferred to developing solution (45gm sodium carbonate + 2 ml formaldehyde + 50 µl of 10% sodium thiosulphate) till bands were developed. The gel was fixed in 8 % acetic acid for 10 min and finally kept in distilled water for 2 min. Up regulated, down regulated, differentially expressed bands and uniformly expressed bands were identified and marked on the plate. Gel was then dried overnight at room temperature.

3.3.5 Elution of cDNA /transcript derived fragments from Poly acryl amide gel

The identified cDNA fragments in the gel were rehydrated for 20 min with 10 µl of distilled water, and scooped out cut from the Poly acryl amide gel with a surgical blade. Each gel slice was incubated in 40 µl of distilled water for 2 h at room temperature (Minsheng, 2006). Elute 10 µl from 40 µl and the eluent 10 µl was then PCR amplified under the same conditions as for the selective PCR reaction with modified dNTP concentration i.e, 2mM. PCR product was separated on a 1.5% agarose gel and purified with an AxyPrep DNA Gel Elution Kit (Axygen, Biosciences) as per the following procedure.

3.3.5.1. Gel elution of PCR amplified fragments

cDNA fragments that were obtained after PCR amplification on gel was eluted using Axy Prep DNA Gel Extraction Kit (Axygen, Biosciences) as per the manufacturer's guidelines.

1. cDNA bands were excised from the gel using a sterile, sharp scalpel avoiding much exposure to UV on a trans illuminator.
2. Gel slice was weighed in a 1.5 ml micro centrifuge tube.
3. Added 3X gel volume of gel solubilization buffer (DEA buffer).
4. The gel was resuspended in gel solubilization buffer by vortexing. The mix was heated at 75^0C until the gel was completely dissolved. Intermittent vortexing was given every 2 to 3 min to enhance gel solubilization.
5. Added 0.5X gel solubilization volume of binding buffer (DEB buffer) and mixed properly.
6. A spin column was placed in a 2 ml collection tube. The solubilized gel slice was transferred into the spin column that was assembled in the 2ml collection tube and centrifuged at 12,000g for 1 min.
7. The filtrate was discarded. Added 500 µl of wash buffer (W1) to the spin column and centrifuged at 12,000g for 30 sec.
8. The filtrate was discarded and 700 µl of desalting buffer (W2) to the spin column and centrifuged at 12,000g for 30 sec.
9. A second wash was given by adding 700 µl desalting buffer (W2), followed by centrifugation at 12,000g for 30 sec to ensure the complete removal of salt. The filtrate was discarded and spin column was again placed in collection tube.
10. Column was again centrifuged for 1 min at 1,2000 g to remove any residual buffer.

11. Spin column was transferred to a fresh 1.5 ml micro centrifuge tube. The elute was pre warmed at 65^0C to improve the elution efficiency.
12. To elute the DNA, 7 µl of eluent was added to the spin column. It was allowed to stand for 1 min at room temperature. Then centrifuged at 12,000 g for 1 min.

The eluted cDNA fragments were checked on 0.7 percent agarose gel and stored -20^0C for further analysis.

3.4. Cloning of cDNA/Transcript derived fragments

The eluted and purified cDNA product was cloned in to pGEMT vector system (Plate 3) Clone GEMT (Fermentas). As per manufactures instructions.

3.4.1. Transformation of *E.coli*

3.4.1. Preparation of competent cells

Competent cells for plasmid transformation were prepared using the competent cell preparation Kit of GeNei, Bangalore in LB agar media. (Chemical compositions of media are given in Annexure IV)

Procedure

Day 1

The desired str

plate. The plates were incubated at 37°C for 16 to 18 hrs.

Day 2

1. Inoculated 5 to 6 moderately sized colonies from the LB agar plate in 50 ml Lb broth in a 500 ml flask

2. Incubated the broth on a rotary shaker at 37^0C; 160 rpm for 4-5 hour. When the OD reached 0.3 at A600, the growth of the bacteria was arrested quickly by chilling for 2 min on ice.
3. The entire culture was transferred into a 500ml sterile polypropylene tube and centrifuged at 3500rpm for 15 min at 4^0C.
4. The supernatant was discarded. Keeping the bottle on ice, the bacterial cell pellet was resuspended very gently in 16.6 ml ice cold Solution A (provided in the Kit).
5. Kept on ice for 20 min and then centrifuged at 3500 rpm for 15 min at 4^0C.
6. The supernatant was discarded and the pellet was chilled on ice.
7. The pellet was resuspended in 3 ml of ice cold Solution A.
8. The suspension was left on ice for 10 min and aliquots of 100 µl were added in chilled 1.5 ml micro centrifuge tubes.
9. The competent cells were covered with aluminium foil and were stored at -70^0C.

3.4.2. Screening of competent cells

The competent cells prepared were screened to check their transformation efficiency, by transforming them using a plasmid (pUC18) containing ampicillin resistance marker. The procedure is as follows:

1. Prepared 50 ml LB broth.
2. The competent cells Kept at -70^0C were thawed on ice.
3. pUC18 was diluted to 1:10 dilution. Added 1 µl of diluted pUC18 to thawed competent cells.
4. The contents were mixed gently and kept on ice for 40 min.
5. Meanwhile, the water bath was set to 42^0C.

6. The tube was rapidly taken from ice and a heat shock at 42^0C was given exactly for 90 sec. Without shaking, the tube was placed back on ice for 5 min.
7. Added 250 µl of LB broth to vial under sterile conditions and was inverted twice to mix the contents.
8. The tube was incubated at 37^0C for 1 hour with shaking.
9. The transformed cells (100, 150, 200 µl) were plated on LB agar/ampicillin (500mg/l) overlaid with IPTG (6 µl) and X gal (60 µl).
10. The plates were incubated overnight at 37^0C.

3.4.3. Ligation

The appropriate amount of PCR product (insert) required for ligation was calculated by estimating the quantity of eluted cDNA using NanoDrop Spectrophotometer. For this, 1 µl of eluted cDNA was diluted to 5 µl with sterile H_2O and then taken reading. The amount of PCR product required was calculated by the following relationship.

$$\frac{\text{ng of vector x kb size of vector}}{\text{kb size of vector}} \text{ X Insert: vector molar ratio}$$

Ligation procedure was followed as per the manufacturer's guidelines.

Procedure

1. The following blunting reaction on ice was set up:

Table 4. Blunting reaction master mix

Component	Volume
2X Reaction Buffer	10 µl
Purified PCR product	1 µl
Water, nuclease-free	To 17 µl
DNA Blunting Enzyme	1 µl
Total volume	18 µl

2. Vortexed briefly and centrifuge for 3-5 s.
3. Incubated the mixture at 70°C for 5 min. Chilled on ice.
4. Set up the ligation reaction on ice. Added the following to the blunting reaction mixture.

Table 5. Ligation reaction

Component	Volume
pGEMT vector (50 ng)	1 µl (0.05 pmol ends)
T4 DNA Ligase	1 µl
Total Volume	20 l

5. Vortexed briefly and centrifuged for 3-5s to collect drops.
6. Incubated the ligation mixture at room temperature (22^0C) for 5 min.
7. Used the ligation mixture directly for transformation.

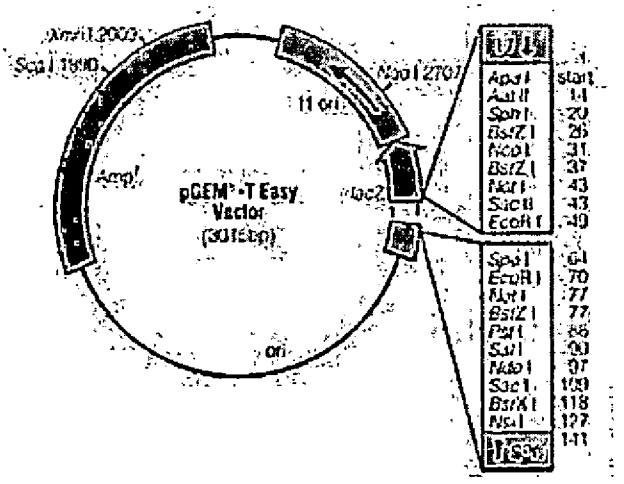


Plate 2. Diagram of pGEM®- T cloning vector

3.4.4. Transformation of ligated product

The aliquots of transformed cells (50, 100 and 150 µl) were plated on LB agar containing Ampicillin (5 mg/ml) plates and incubated overnight at 37^0C.

3.4.5. Analysis of recombinant clones

3.4. 5.1. Colony PCR

1. The following protocol was used for colony screening by PCR
2. Mixed well and the transferred 20 µl of the mix into the PCR tubes on ice.
3. Picked an individual colony and resuspended in 20 µl of the PCR master mix.
4. Performed PCR reaction as per the thermal program in table 7.
5. Analyzed on 1% agarose gel for the presence of the PCR product.

Table 6. Master mix for colony PCR

Component	Volume/reaction (µl)
10X tac buffer	2
dNTP(10mM)	2
$Mgcl_2$	1.2
pGEMT forward sequencing primer(10 µM)	0.4
pGEMT reverse sequencing primer(10 µM)	0.4
Tac polymerase (5u/µl)	0.1
Water(Nuclease free)	13.9
Total	20

Table7. Temperature profile for colony PCR

S.No	Step	Temperature	Time
1	Initial denaturation	95^0 C	3 min
2	Denaturation	94^0C	30s
3	Annealing	60^0C	30s
4	Extension	72^0C	1 min
5	Step 2 to 4	30cycles	
6	Final Extension	72^0C	10 min
7	Hold	4^0C	10 min

3.4.5.2. Preparation of stabs

Stabs were prepared for the colonies in which the presence of insert was confirmed. LB agar medium was melted, cooled to 42^0C, added ampicillin and poured into storage vials aseptically in a laminar air flow. After solidification, single colony was inoculated into the medium using sterile bacterial loop. The vials were incubated at 37^0C overnight and further stored at 4^0C.

3.5. Sequencing of cDNA clones

The up regulated, down regulated and differentially expressed transcript derive fragments were cloned in pGEMT and sequenced using specific forward and revers primer through SciGenome, Cochin.

3.5.1. *In silico* analysis of sequences

The sequences obtained were analyzed with the following online bioinformatic tools and validated.

1. **Vector screening**

   To remove the vector regions present in the sequences, vecto screening was performed using VecScreen too (www.ncbi.nlm.nih.gov/VecScreen) provided by NCBI. The vector and th adopter sequences present were removed using Bioedit-Biological sequenc alignment editor tool.

2. **Reverse complement of sequences**

   To merge the sequences generated by forward and reverse primer, on of the sequences has to be reverse complemented. This was done by usin online tool 'Reverse complement' available on line.

3. **Merging of sequences**

   Sequences generated by forward primer and reverse complemente sequence of reverse primer merged by using online tool 'Emboss Merger available online at http://www.bioinfo.nhri.org.tw/gui/.

4. **Search for Homolog**

   The nucleotide sequence of all the sequences were compared with th sequences available in nucleotide database using BLAST too (www.ncbi.nlm.nih.gov/Blast/; Altschul *et al.*, 1997) provided by NCBI Nucleotide- Nucleotide sequence comparision was done using Blastn an Nucleotide- Protein was done by using Blastx. The best sequence alignmen results were noted and saved.

Results

4. RESULTS

The present study entitled "Molecular characterization of black pepper (*Piper nigrum* L.) genotypes for drought tolerance" was carried out at Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University, Vellanikkara during the period from 2013-2015.

4.1. Physiological studies for screening of drought tolerant black pepper genotypes

The physiological parameters were measured to evaluate the black pepper genotypes for drought tolerance under water stress conditions. Relative water content, membrane integrity and number of days taken for wilting were measured at 5 days interval up to 15 days of stress induced plants. Statistical analysis was carried out using ANOVA and there was a significant difference at 5% level by DMRT.

4.1.1. Relative water content

Relative water content in all the ten black pepper genotypes were decreased significantly from fifth day to fifteenth day of stress induction. Under stress, maximum relative water content was observed in Angamaly (PRS-64) which was on par with PRS-155 and PRS-160. The minimum relative water content was observed in Panniyur-1 (PRS-44) which was on par with Karimunda (Table7).

4.1.2. Membrane Integrity

Membrane Integrity decreased with increase in stress intensity. It was calculated by measuring electrical conductivity using EC meter. The highest membrane integrity was observed in Angamaly which was on par with Panniyur-8, Panniyur-5 and PRS-161. The lowest membrane integrity was observed in Panniyur-1 which was on par with PRS-156 (Table8).

4.1.3. Days taken for wilting

The genotype Panniyur-1 shows early wilting at thirteenth day of stress induction (Plate 2) which was on par with PRS-156. Angamaly shows late wilting at twenty third day which was on par with Panniyur-8 (Table 9) (Plate 4).

Table7. Effect of water stress on Relative water content of pepper genotypes

Pepper genotypes	5 DASI	10 DASI	15 DASI
PRS - 115 (Panniyur-5)	65.6^{e}	47.5^{cd}	17.5^{c}
PRS -149 (wild type)	65.4^{e}	42.2^{ef}	26.7^{b}
PRS -155 (wild type)	81.7^{a}	65.7^{a}	26.4^{b}
PRS - 64 (Angamaly)	82.8^{a}	67.8^{a}	31.5^{a}
PRS - 153 (Panniyur-8)	76.1^{bc}	46.5^{cde}	18.2^{c}
PRS -156 (*Piper attenuatum*)	76.1^{bc}	46.6^{cde}	18.2^{c}
PRS - 44 (Panniyur-1)	70.2^{de}	43.5^{def}	12.5^{de}
PRS - 160 (Hybrid)	80.0^{ab}	54.4^{b}	16.1^{cd}
PRS - 161 (Hybrid)	76.0^{bc}	51.2^{bc}	25.6^{b}
Karimunda	72.2^{cd}	41.4^{f}	12.1^{e}
CV	4.814	6.787	12.95
CD	5.187	4.967	3.832

a;b;c;d;e;f- (Value followed by different alphabets indicate significant difference) Duncan score of the DMRT analysis. .DASI-Days after stress induction)

Plate 3: Control plants

Plate 4: Water stress imposed plants

Table 8. Effect of water stress on Membrane Integrity of pepper genotypes

Pepper genotypes	5 DASI	10 DASI	15 DASI
PRS -115 (Panniyur-5)	92.3^{a}	88.8^{b}	87.0^{ab}
PRS –149 (wild type)	89.5^{cd}	84.2^{c}	77.1^{de}
PRS - 155 (wild type)	88.8^{de}	81.9^{d}	76.0^{e}
PRS - 64 (Angamaly)	93.0^{a}	90.7^{a}	89.4^{a}
PRS -153 (Panniyur-8)	92.6^{a}	90.0^{ab}	88.1^{ab}
PRS - 156 (*Piper attenuatum*)	89.8^{cd}	82.4^{d}	72.7^{f}
PRS - 44 (Panniyur-1)	87.5^{e}	81.5^{d}	71.8^{f}
PRS – 160 (Hybrid)	90.6^{bc}	85.1^{c}	81.9^{c}
PRS - 161 (Hybrid)	91.5^{ab}	84.8^{c}	86.2^{b}
Karimunda	89.0^{d}	84.9^{c}	79.1^{d}
CV	1.166	1.314	2.322
CD	1.524	1.622	2.714

a;b;c;d;e;f- Duncan score of the DMRT analysis

Table 9. Number of days taken for wilting by different pepper genotypes

Pepper genotypes	Days taken for wilting
PRS - 115 (Panniyur-5)	20^{bc}
PRS - 149 (wild type)	17^{de}
PRS - 155 (wild type)	16^{ef}
PRS - 64 (Angamaly)	23^{a}
PRS -153 (Panniyur-8)	21^{ab}
PRS - 156 (*Piper attenuatum*)	14^{fg}
PRS - 44 (Panniyur-1)	13^{g}
PRS - 160 (Hybrid)	18^{cde}
PRS - 161 (Hybrid)	19^{bcd}
Karimunda	17^{de}
CV	9.62
CD	2.473

a;b;c;d;e;f;g- Duncan score of the DMRT analysis

PRS-64 identified as most drought tolerant genotype and PRS-44 identified as susceptible genotype based on RWC, MI and days taken for wilting.

4.2. Molecular characterization of drought tolerant genotype

4.2.1. Isolation and analysis of total RNA

Using trizol reagent total RNA was isolated from two treatments. The samples were run on 2 percent agarose gel in 1X MOPS buffer. Three intact bands corresponding to 28s, 18s, and 5S rRNA + tRNA were obtained (Plate 5), indicating the quality of RNA. The ratio of OD_{260}/ OD_{280} for the samples was greater than 1.8 indicating pure RNA free from DNA and protein contamination. The ratio for OD_{260}/ OD_{230} was greater than 1.0 indicating that the samples were free from polysaccharides and polyphenols.

4.3. Differential Display Reverse Transcription Polymerase Chain Reaction (DD-RT-PCR)

About 1 µg of total RNA from all the treatments were taken for DD-RT- PCR analysis. The first strand cDNA was synthesized from the above RNA samples HT11G (AAGCTTTTTTTTTTTG). Each first strand cDNA was used for the second strand amplification with 8 different arbitrary primers as described in table no.1. The PCR product was resolved on 6% denaturing urea poly acrylamide gels and visualized after silver staining (Plate 6). The gel was dried overnight for elution and further analysis.

4.4. Elution and reamplification of differently expressed cDNAs

The up regulated, down regulated and differentially expressed cDNA fragments were retrieved from the gel and reamplified with the same set of primers as

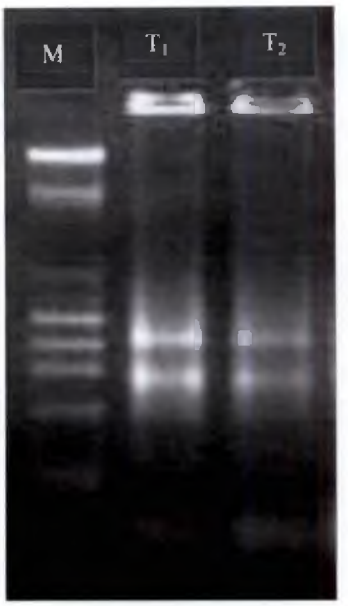


Plate 5. Agarose gel electrophoresis showing total RNA isolated from different treatments (M-Marker λ DNA/ EcoRI + Hind II

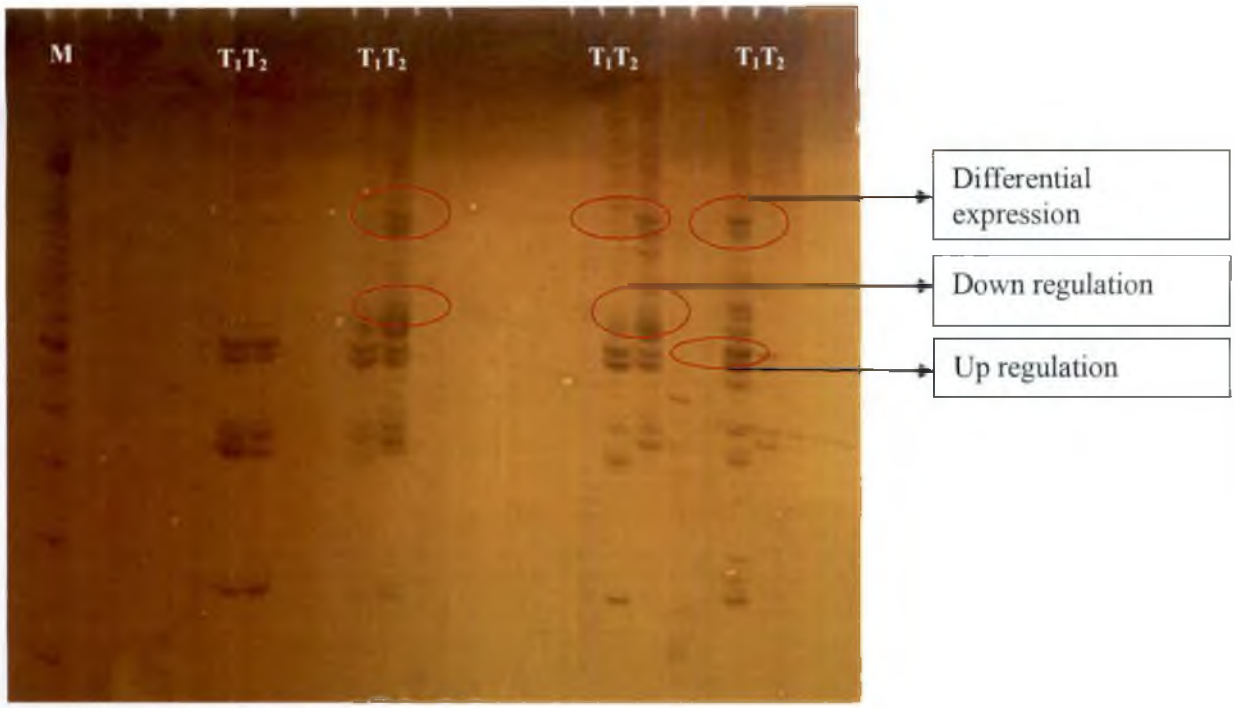


Plate 6. Differential display patterns of transcript derived fragments from two treatments in PRS-64 amplified using anchored primer H-T11 (G) and arbitrary 13-mers (HAP-1 to 4)

in the initial DD-RT-PCR reaction and analysed electrophoretically. The agarose gel electrophoresis showed that the cDNA fragment obtained were relatively short (300-900bp).

4.5. Gel elution of PCR amplified fragments

cDNA fragments that were obtained after PCR amplification on gel was eluted using AxyPrep DNA Gel Extraction Kit (Axygen, Biosciences). The quantity estimated was 0.6ng cDNA/ µl based on absorbance at 260 nm and 280 nm.

4.6. Cloning of transcribed derived fragments (TDFs)

4.6.1. Preparation and screening of competent cells

The competent cells prepared were checked for competence by transforming the plasmid (pUC 18) having ampicillin resistance. A large number of blue colonies were obtained which indicated a high degree of transformation efficiency. Thus the competent cells prepared were found ideal for cloning amplicons.

4.6.2. Ligation

The amount of PCR product (insert) required for ligation was calculated thus, 30ng of insert was used per 50 ng of vector ligation reaction using pGEM T vector, considering the fact that the eluted sample had 0.6 ng cDNA/µl.

4.6.3. Transformation of ligated product

The prepared competent cells was used to transform the ligated product and incubated at 37^0 C. Large number white colonies were obtained after overnight incubation when the transformed cells were cultured in LB/ampicillin media.

4.7. Screening of the transformed colonies

The pGEM T vector used in the present study for cloning which gives white colonies for recombinant plasmid and blue colonies for non-recombinants (Plate 7). The plasmid DNA isolated from white colonies when viewed after electrophoresis in 0.8 percent agarose gel showed greater plasmid size due to the presence of insert in the plasmid (Plate 8). The recombinant plasmids harboring cloned TDFs were sequenced (Sci Genome Pvt Ltd, Cochin). The sequencing results showed that the partial cDNA clones are of size between 300 to 900 bp. All the cDNA were found to possess poly A tail (corresponding to oligo dT primer) at 3' end and the random arbitrary primer sequence at the 5' end.

4.8. Sequence data analysis

The nucleotide sequences of cDNA fragments were annoted with the NCBI database using the NCBI-BLAST (n and x) program to determine homologous protein.

4.9. Functional annotation of functional genes

Analysis of the cDNA sequences against NCBI database revealed that cDNAs were found to belong to the category of genes related to R genes, NADH dehydrogenase, heat short proteins, protein kinases and transcription factors.

Some of the proteins were hypothetical which belonged to the category whose functions were not yet identified. The details of result obtained are presented below.

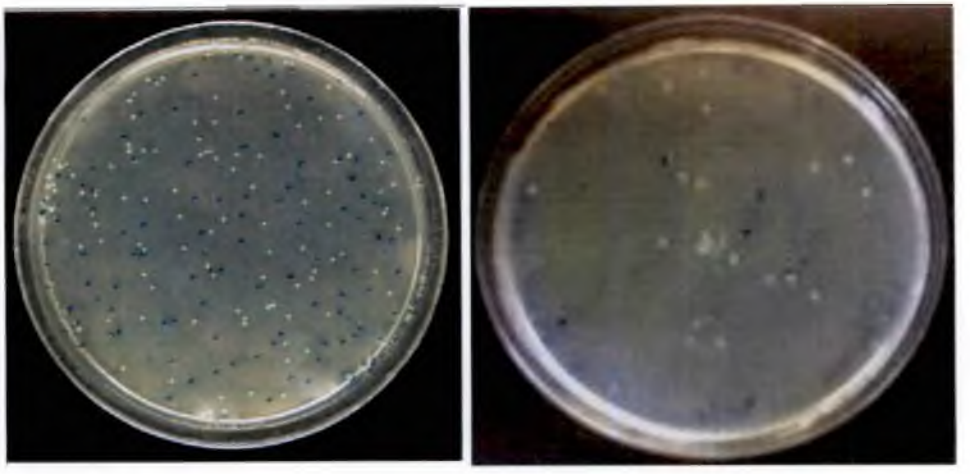
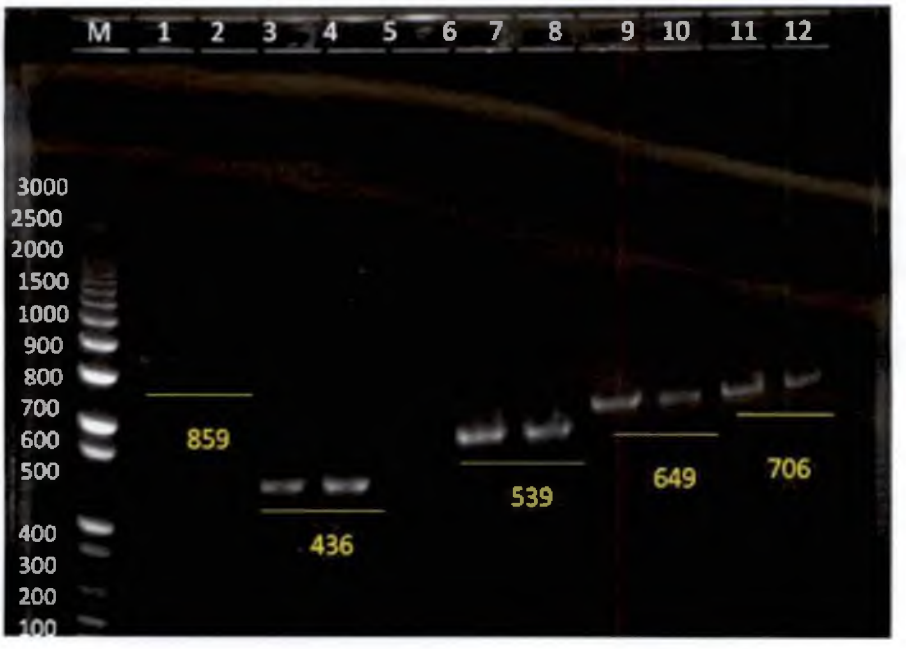


Plate 7: Cloning of recombinants



M- Marker(100bp) Lane 1-2: Clone 1 Lane 2-4: Clone 2
Lane 6-7: Clone 3 Lane 8-9: Clone 4 Lane 10-11: Clone 5

Plate 8: Confirmation of insert by colony PCR

Clone 1.

The sequence data obtained for the clone 1 with reverse primer (pGEM T) was 907 bp in size and vector sequence indicated vector from 680-728 (Fig. 1.). The trimmed sequence was 859 bp. The details of BLAST n and BLAST x results are given in fig.2-3 and table 10-11. Clone 1 shows homology to NADH dehydrogenase with maximum identity.

Clone 2.

The sequence data obtained for the clone 2 with forward primer (pGEM T) was 463 bp in size and vector sequence indicated no vector sequence (Fig.4). Details of BLAST n results are given in fig.2 and table 10 and BLAST x results are given in fig.5-6 and table 12-13. Clone 2 shows homology to calcium-dependent protein kinase with maximum identity.

Clone 3.

The sequence data obtained for the clone 3 with forward primer (pGEM T) was 590 bp in size and vector sequence indicated vector sequence from 1-82 and 361-384 (Fig.7).The trimmed sequence was 539 bp. Details of BLASTn result are given in Fig.8 and Table 14. Details of BLASTx result are given in fig.9 and table 15. Clone 3 shows homology to ribosomal RNA gene with maximum identity.

Clone 4.

The sequence data obtained for the clone 5 with forward primer (pGEM T) was 669 bp in size and vector sequence indicated vector sequence from 650–669 bp (Fig.10). The trimmed sequence obtained was 649 bp.

Clone 1 (859 bp)

5`tccgaatgcaacaaaaaatcttcgccgcatgtgggcttttattagtgttttattattaagcatagctttccttttttcgatcgatatatccat
tcaacaaataagaggcagctccgtttgtcaatatctatggtcttggaccatmaataatgatttttctttggagtttggmtacttgattgatt
csettacttctattatgttaatactaatcactactgttggaatcatggttcttatttatagtgataattacatgtctcatgaccaaggatatttga
gatttttttgcgtatcttaatttttttcaatgcttccatgcttggattagttactagttccaatttaatacaaatttatatttttttgggaacttgtagga
atgtgctcttatttactaataggtttttggttcacacgacccattgcagcaaatgcttgtcaaaaagcctttgtaactaatcgtgtagggga
ttttggtttattattaggaatcttaggactttattggataacgggcagttttgaatttcgggatttatgccttaaaatccagatcaagggaagt
ctccaatgaggacacaaaaaccagaagccgatggggcggtggtggtcgcagcgggaatttttcttgttgctcgacttttcctcttttta
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ccattattcattctatggaac 3`

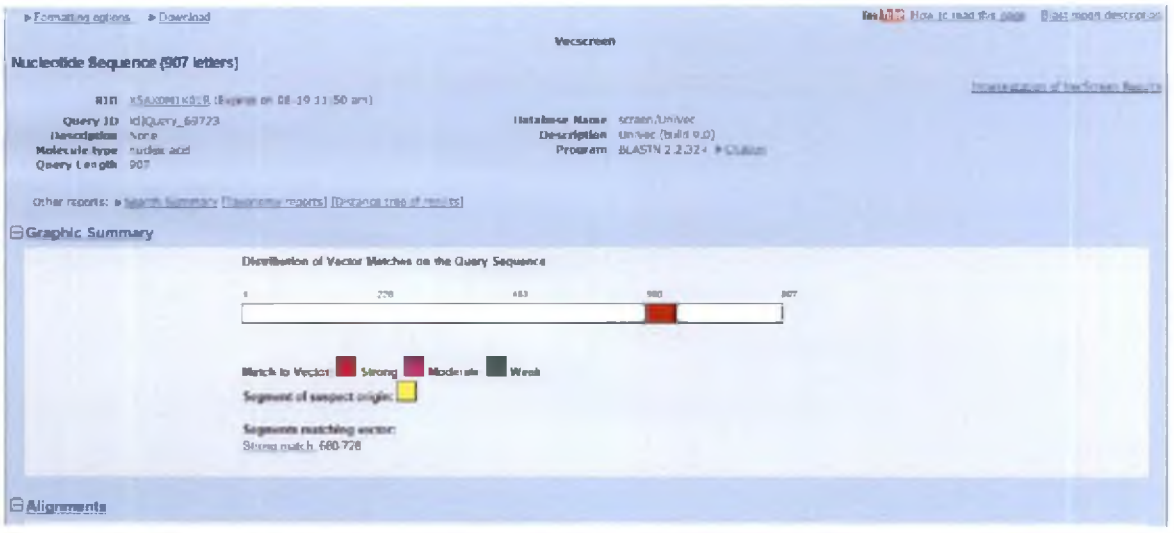


Fig.1. Vecscreen diagram of clone 1 with reverse primer

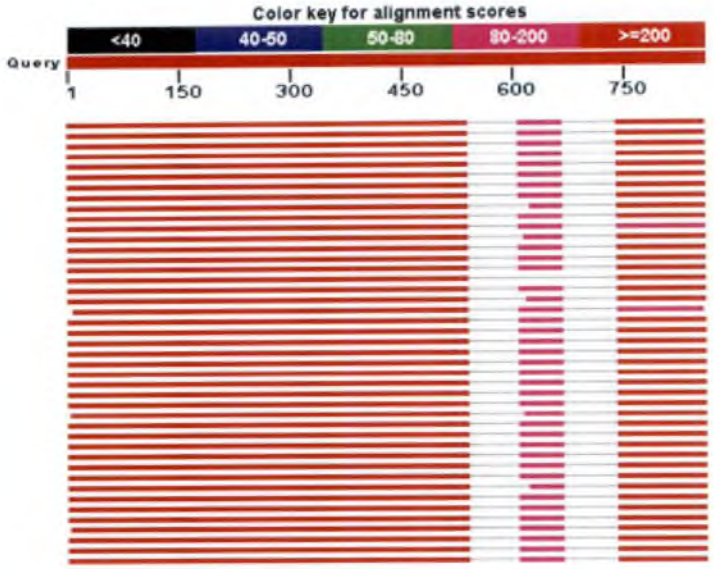


Fig.2. Blastn analysis of clone 1

Table 10. Blastn analysis of clone 1

Accession	Description	Max. score	Query coverage(%)	E Value	Max. Identity(%)
DQ356472.1	*Piper nigrum* NADH dehydrogenase subunit F (ndhF) gene, partial cds: chloroplast	985	83	0.0	100
EU519671.1	Piperquineense voucher J smith 4924 NADH dehydrogenase F subunit-like (ndhF) gene	963	83	0.0	99
EU519730.1	Piperaequale voucher A Bornstein 716 NADH dehydrogenase F subunit-like (ndhF) gene, partial	935	83	0.0	98
EU5197061.1	Piper muricatum voucher W Wong 2 NADH dehydrogenase F subunit-like	935	83	0.0	98

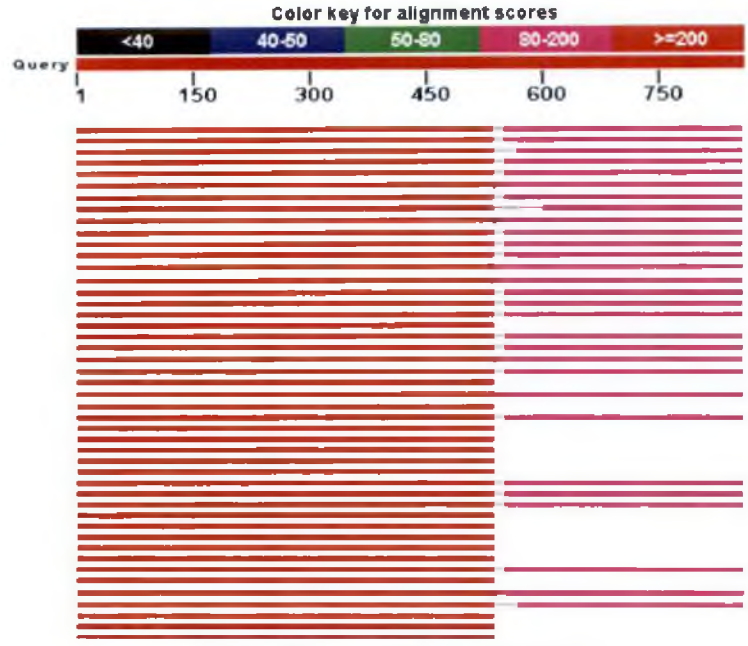


Fig.3. Blastx analysis of clone 1

Table 11. Blastx analysis of clone 1

Accession	Description	Max. score	Query coverage(%)	E value	Max. Identity(%)
ABC79698.2	NADH dehydrogenase subunit F(*Piper nigrum*)	345	98	1e-112	100
ACC91320.1	NADH dehydrogenase F subunit (*Piper methysticum*)	329	98	5e-112	95
ACC91323.1	NADH dehydrogenase Fsubunit (*Piper puberulum*)	336	96	5e-112	97
ACC91321.1	NADHdehydrogenase Fsubunit (*Piper amalago*)	331	98	1e-106	97

Clone 2 (463bp)

5`acgcgggggtgcgaagcatatgaatagtagaagaatgtgaagtcacaaacaaacactgactctgactcttttgaacttccacca
gtaaaacctcttgccatctccacccatattaagataagcaatgctcgttccatcggcttctggttttgtgtcctcattggagacttcccctt
gatctggattttaaggcggtggaggaaacccgttgtgaagaatccccaattggttcccaaccctaaaaccccccacgggtttctgttc
atttggttatggagctttgtgaaggtggtgagttgtttgataggattgtgaacaagggggccatggccgcgggattagcgtggtcgc
ggccgaggtcattatagtgagagggaagctgctaagctgattaggactattgtcgaggttgtggaaaattgtcattctcttggtgttat
gcatagggaccttaagccggaga 3`

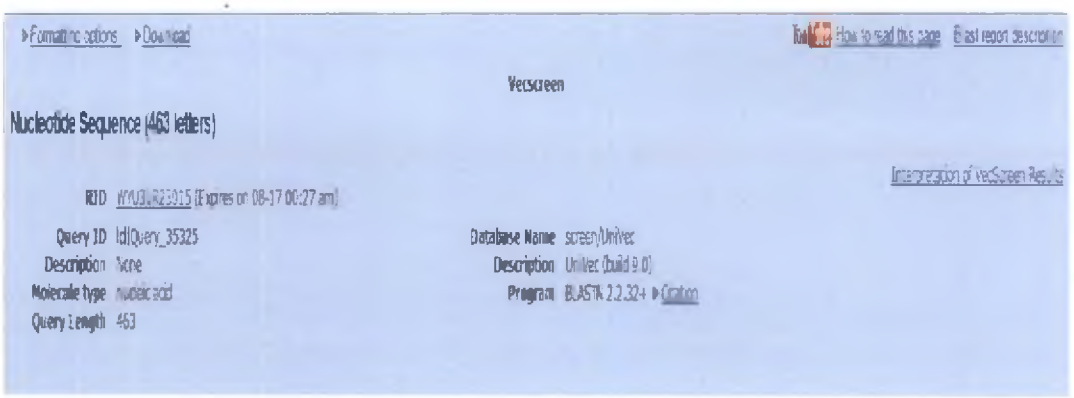


Fig.4. Vecscreen diagram of clone 2

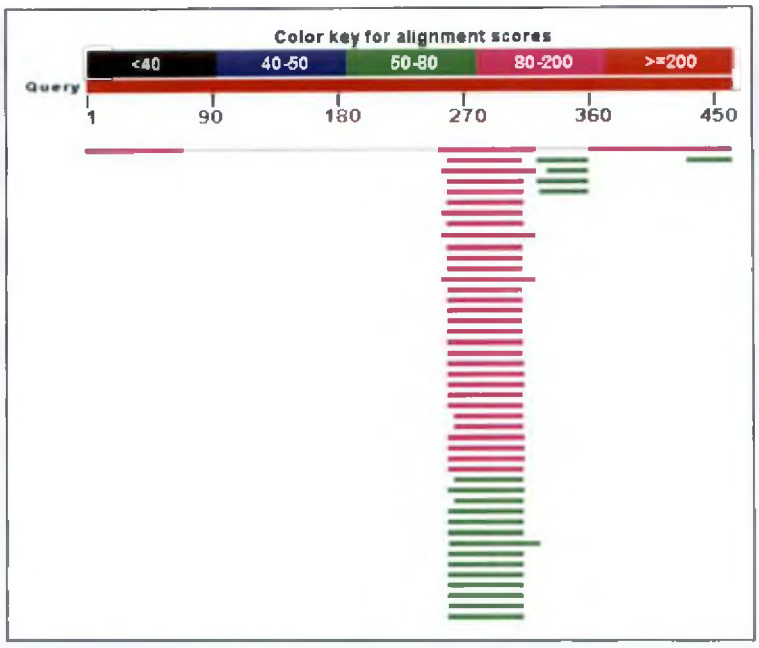


Fig. 5. Blastn analysis of clone 2

Table 12. Blastn analysis of clone 2

Accession	Description	Max. score	Query coverage(%)	E value	Max. Identity(%)
AAV28169.1	Calcium-dependent protein kinase 1(*Vicia faba*)	73.2	36	1e-19	100
XP0045030201.1	PREDICTED: Calcium-dependent protein kinase SK5(*Cicer arietinum*)	70.1	36	4e-18	94
XP 007137839.1	Hypothetical protein PHAVU 009G160100g(*Phaseolus vulgaris*)	69.3	36	6e-18	91
XP 012459667.1	Calcium-dependent protein kinase 2 isoform 1(*Theobroma cacao*)	68.2	37	6e-18	91

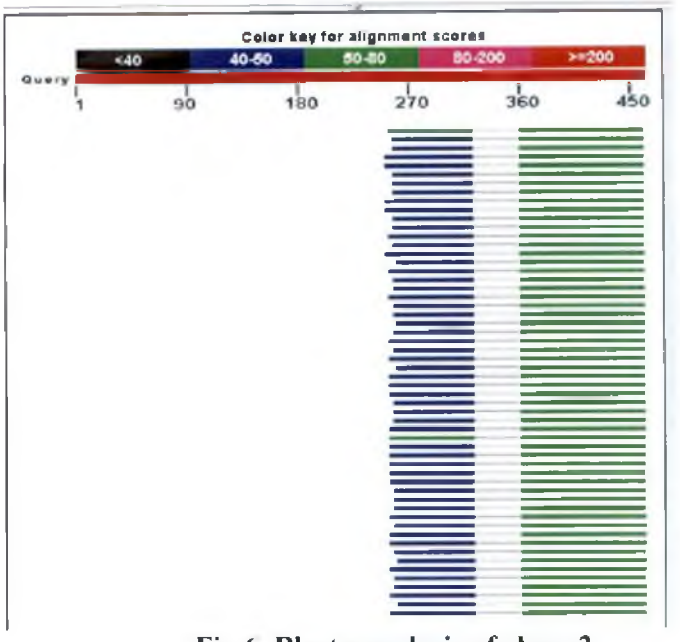


Fig.6. Blastx analysis of clone 2

Table 13. Blastx analysis of clone 2

Accession	Description	Max. score	Query coverage(%)	E value	Max. Identity(%)
AY753552.1	*Vicia faba* calcium-dependent protein kinase 1 (CPK1) mRNA complete cds	191	452	1e-44	100
XM010429330.1	*Camelina sativa* Calcium-dependent protein kinase32-like (LOC1047124281)	102	102	5e-18	100
XM 004502963.2	*Cicer arietinum* calcium-dependent protein kinaseSK5(LOC101512548) m RNA	990	11	7e-17	93
XM009118207.1	PREDICTED: *Brassica rapa* calcium-dependent protein kinase32	990	14	7e-17	98

Clone 3 (539)

5`ttatgcgatacttggtgtgaattgcagaatcccgtgaaccatcgagtctttgaacgcaagttgcgcccgaggcctttggtcgagggcacatctgctt
gggcgttaaacaactcgcctcccgccgccttcccccctgcagactcgagcgaggggcctgtcggagatgagggtaggtgggacggagtctggtcgt
ccgtgtgcctgcagcgcatgcggttggctgaaaagcctgggccatgggctgcgtgcggctcagcgagtggtggttgtgcgccctcgcaaggcggc
gatcgtcggcgcacctgcccgggcggccgctcgagcgtgggagggttgtgccgttcgctcccgattgcccggcaagtacccgacacgatcgaaa
catccaatccacggatcgattcgaatttgcaaccccaagtcaggtgggactacccgccgagcttaagcatatcaataagcggaggaatcctagattca
agtgaagtctccaatgaagaacacaaaaacagaagccgattgaaacgagcgctcccg3`

Fig.7. Vecscreen diagram of clone 3 with reverse primer

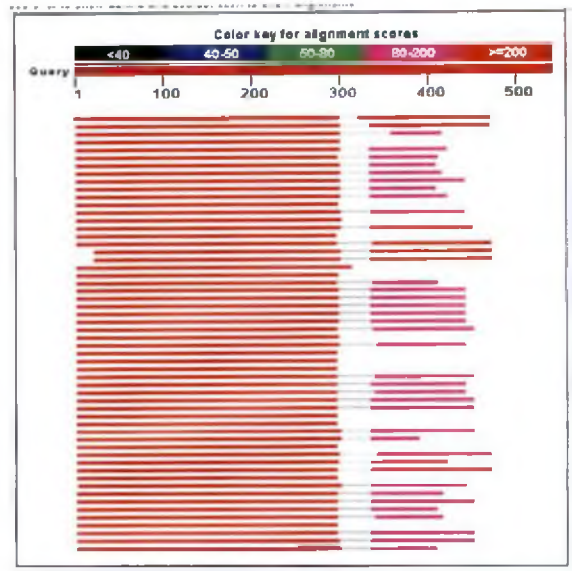


Fig.8. Blastn analysis of clone 3

Table 14. Blastn analysis of clone 3

Accession	Description	Max. score	Query coverage(%)	E value	Max. Identity(%)
KP406137.1	*Piper nigrum* 5.8 ribosomal RNA gene, partial sequence: internal transcribed spacer 2	555	83	4e-154	100
JF491442.1	*Piper nigrum* isolate HJZ 18s ribosomal RNA gene, partial sequence: internal transcribed spacer	540	80	1e-149	99
AM901428	*Piper nigrum* ITS1, 5.8S rRNA gene and ITS2, specimen Voucher Menezes	538	66	4e-149	99
KF924124.1	*Piper nigrum* isolate HCY-38 18S ribosomal RNA gene, partial sequence	534	56	5e-148	99

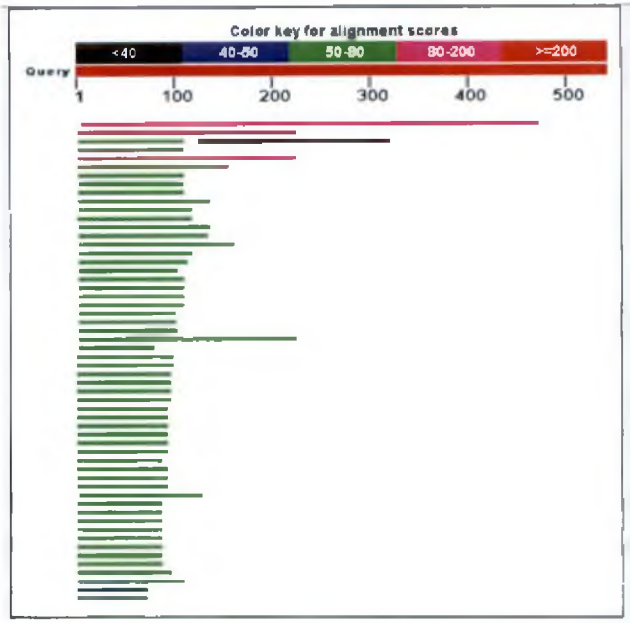


Fig.9. Blastx analysis of clone 3

Table 15. Blastx analysis of clone 3

Accession	Description	Max. Score	Query coverage(%)	E value	Max. Identity(%)
CDY55729.1	Hypothetical protein BVRB 017900 (*Beta vulgaris subsp. Vulgaris*)	82	86	3e-16	41
XP 006380095.1	BnaC08g46150D (*Brassica napus*)	84	41	1e-15	61
XP006375694.1	Hypothetical protein POPTR 0008s21840g (*Populus trichocarpa*)	76.6	20	8e-15	89
CDY66372.1	Hypothetical protein POPTR 00014s19710g (*Populus trichocarpa*)	76.6	20	9e-15	89

Clone 4 (649 bp)

5`cccgcaaacctggagtctgcactaggcaggcctcaagtctcggatgagaatgaggacgagaagaataagacggaatccttgaac
gagaagtttgagggcttatgcatggtgatcaaggatgtcttgggagataaggtggagaaagttgtagtttctgaccgtgtggtggactc
cccctgttgtttggtaactggtgagtatggttggactgccaacatggagaggatcatgaaggcccaggcactgagggactctagcatg
gcgggatatatgtcttccaaaaagactatggagatcaaccccgagaatgcgatcatggaggagctcagaaagagggcagatgctga
caagaacgacaagtccgtgaaagatttggtgctgctgctcttcgagtaccccaatgattacattgcgcccgaggccttttggtcgaggg
cacatctgcttgggcgttaaacaactcgcctcccgccgccttcccctgcagactcgagcgaggggcctgtcggagatgagggtagg
tgggacggagtctggtcgtccgtgtgcctgcagcgcatgcggttggctgaaaagcctgggccatgggctgcgtgcggctcagcgag
tggtggttgtgcgccctcgcaaggcggcgatcgtcggcgc 3`

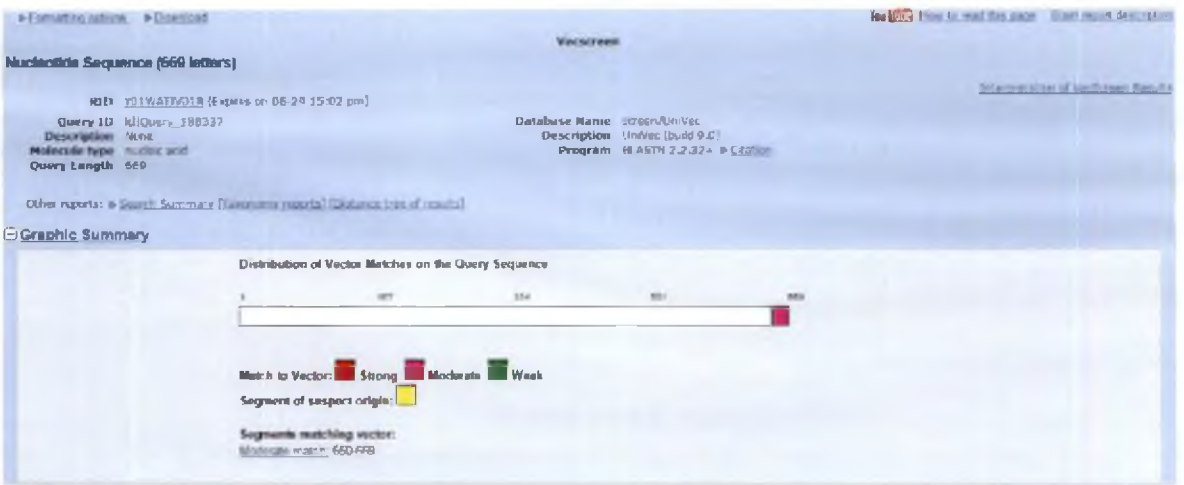


Fig.10. Vecscreen diagram of clone 4 with reverse primer

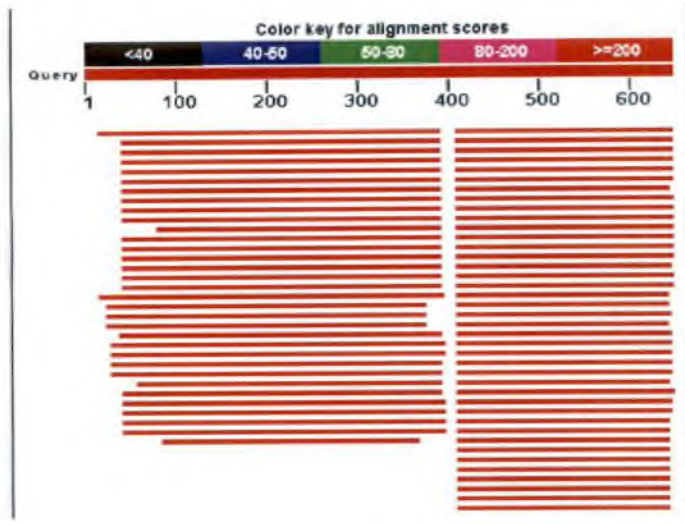


Fig.11. Blastn analysis of clone 4

Table 16. Blastn analysis of clone 4

Accession	Description	Max. score	Query coverage(%)	E value	Max. Identity(%)
KP406137.1	*Piper nigrum* 5.8 ribosomal RNA gene, partial sequence: internal transcribed spacer 2	444	36	1e-120	100
AM901428.1	*Piper nigrum* ITS1, 5.8S rRNA gene and ITS2, specimen Voucher Menezes ICD C18	438	36	5e-119	99
AM901430.1	*Piper nigrum* ITS1, 5.8S rRNA gene and ITS2, specimen Voucher Menezes ICD C11	435	36	7e-118	99
JF491442.1	*Piper nigrum* isolate HJZ 18s ribosomal RNA gene, partial sequence: internal transcribed spacer	433	36	2e-117	99

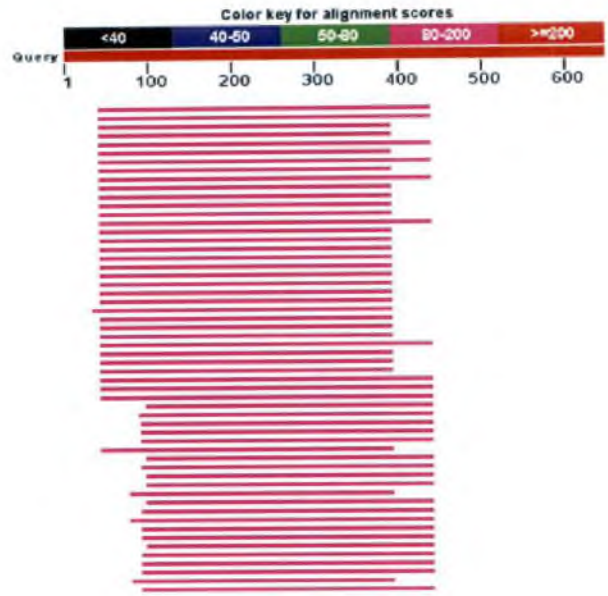


Fig.12. Blastx analysis of clone 4

Table 17. Blastx analysis of clone 4

Accession	Description	Max. score	Query coverage(%)	E value	Max. Identity(%)
EEC84770.1	Hypothetical protein OsI 31800 (*Oryza sativa indica* group)	187	61	3e-55	80
AGT98548.1	Heat shock protein 90 (*Paeonia lactiflora*)	187	61	2e-54	79
KMZ57743.1	Heat shock cognate protein 80 (*Zostera marina*)	192	54	3e-53	93
CAN62488.1	Hypothetical protein VITISV 029391 (*Vitis vinifera*)	191	54	6e-53	93

Details of BLAST n result are given in Fig.11 and Table 16. Details of BLASTx result are given in fig.12 and table 17. Clone 4 shows homology to ribosomal RNA gene with maximum identity.

Clone 5.

The sequence data obtained for the clone 4 with reverse primer (pGEM T) was 706 bp in size and vector sequence indicated no vector sequence (Fig. 13.).

Details of BLASTn result are given in fig.14 and table 16. Details of BLAST x result are given in fig.15 and table 17. Clone 5 shows homology to copper amine oxidase1 with maximum identity.

NADH dehyrogenase subunit F gene, calcium-dependent protein kinase1, ribosomal RNA gene, copper amine oxidase1 are differentially expressed genes and hypothetical protein OsI 31800 is up regulated gene

Clone 5 (706 bp)

5`tatgcgtgggtccgcgggcgtaggactgttgcaaattatgagtatggattctattggcatgccatggccgcgggattagcgtggtcgcgg
ccgaggtttctatcaggatggaaaaatagaagctgaagtgaaattgactggaatccttagcttagggcattgcagcctggtgaagttagaaa
gtatggaacaactatagcaccggggctttatgcacctgttcatcagcatttctttgttgctcgcatggacatggaggcgaatttccgaatcaaa
atcctcgtgtaatgagggactagcaacatgggtcaaacaaaatcgaccgctcgaggaagcagatattgttctgtggtatgtgtttggtctgac
acacattcctcgtctcgaagactggcctgtcatgccagtggagcacattggatttatgcttatgccgcatggtttcttcaactgctccccagcc
gtggacgtcccacccagcgccagtgacgctgatgttaaggagaatggagacccgaagcctcccggcggcctcttgtccaagctctagaa
atgttttcttttccttgcatgtctgtagaccacatgtatccaactgtttctgttgtctgcttgatcatcgaacctctgtttcatctctgttttgaagct
tggagttttatttgatgaagtctttcacgtgcacctgccggcggccgcccgta 3`

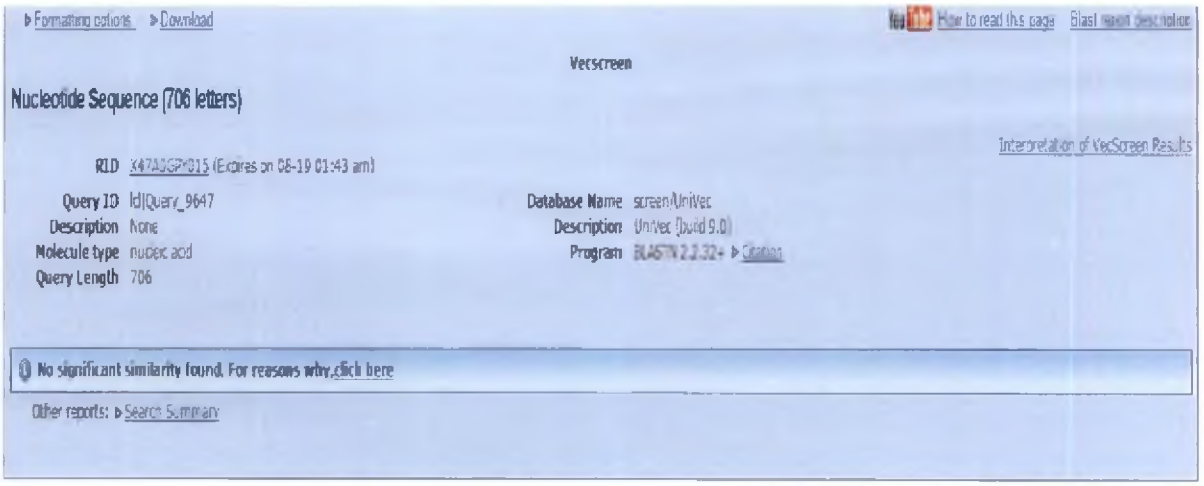


Fig.13. Vecscreen diagram of clone 5

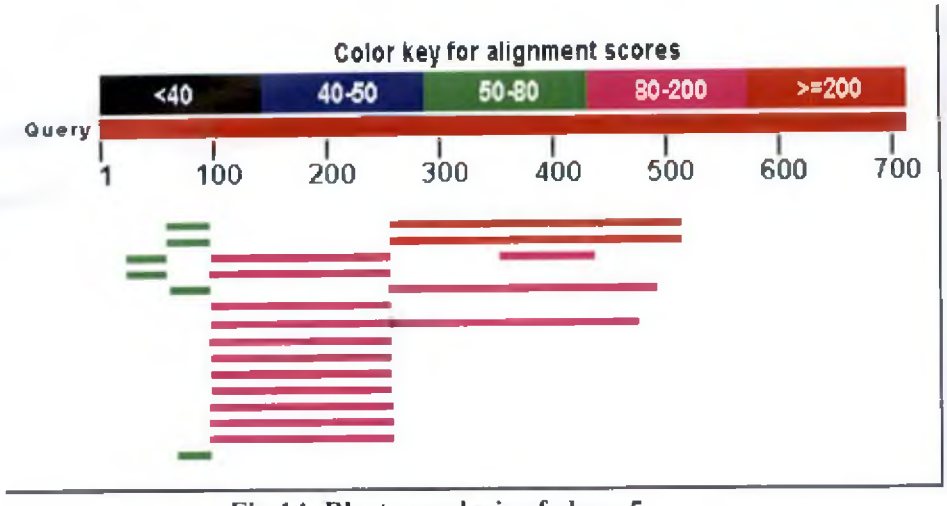


Fig.14. Blastn analysis of clone 5

Table 18. Blastn analysis of clone 5

Accession	Description	Max. score	Query coverage(%)	E value	Max. Identity(%)
XM010648177.1	PREDICTED: *Vitis vinifera* copper amine oxidase1,transcript variant X2	207	36	2e-49	81
XM002273496.3	PREDICTED: *Vitis vinifera* copper amine oxidase1,transcript variant X1	207	36	2e-49	81
XM009405346.1	PREDICTED: *Musa acuminate subsp. Malaccensis* copper amine oxidase 1-like	167	22	3e-37	86
XM10909350.1	PREDICTED: *Elaeis quineensis* copper amine oxidase1, mRNA	163	22	4e-36	85

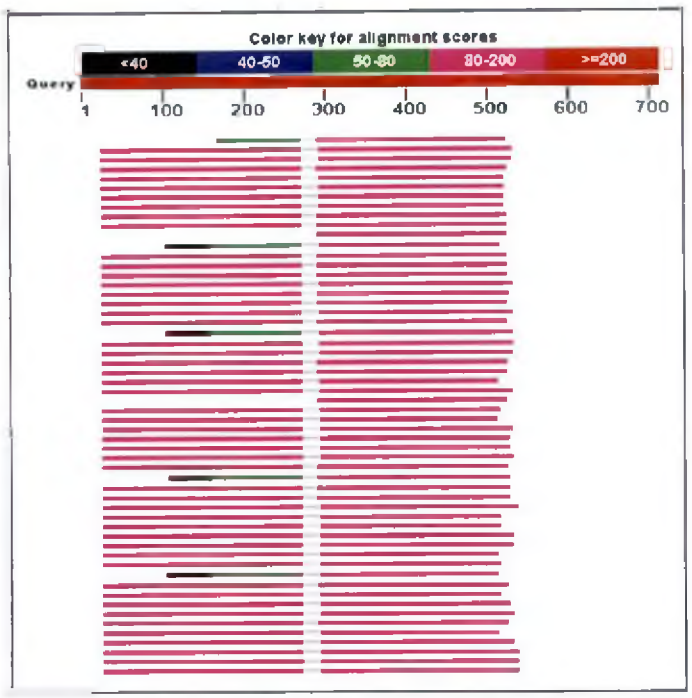


Fig.15. Blastx analysis of clone 5

Table 19. Blastx analysis of clone 5

Accession	Description	Max. score	Query coverage(%)	E value	Max. Identity(%)
CAN 27241	Unknown (*Zea mays*)	148	48	9e-40	88
XP 004509661.1	PREDICTED: Peroxisomal primary amine oxidase-like isoform X1(*Cicer arietinum*)	153	68	7e-39	90
XP 012573826.1	PREDICTED: Peroxisomal primary amine oxidase-like isoform X2 (*Cicer arietinum*)	153	68	8e-39	90
NP 001169	Uncharacterized protein LOC100383438 (*Zea mays*)	148	67	1e-38	88

Discussion

5. DISCUSSION

Drought stress limits the growth and productivity of crops, particularly in arid and semi-arid areas. The availability of water is the major factor influencing the growth, development and productivity. Relative water content is considered a measure of plant water status, reflecting the metabolic activity in tissues. It is used as an index for dehydration tolerance. RWC of leaves is higher in the initial stages of leaf and reduces as the dry matter accumulates. A decrease in the RWC in response to drought stress has been noted in wide variety of plants as reported by Nayyar and Gupta (2006). The electrolyte leakage technique to evaluate the cell membrane stability as a mechanism of water stress tolerance in durum wheat and this method is used based on *in vitro* desiccation of leaf tissues by a solution of polyethylene glycol (PEG) (Lutts *et al.,* 2001). India is the major producer and consumer of Black pepper in the world but our productivity is low (442 kg/ha) compared to other countries especially Thailand (3482 kg/ha) (Thankamani and Babu, 2014). The period (October-May) is considered as water stress period and 20-30 % decrease in yield of black pepper was reported (Vasantha, 2008).

Pepper research station, Panniyur is maintaining germplasm accessions of black pepper genotypes under field conditions. Screening and evaluation of genotypes were carried out to identify genotypes showing tolerance to water stress, under field conditions. Based on the phenotypic parameters 15 genotypes were identified at PRS, Panniyur. Among them ten promising genotypes were further taken for the present study to characterize the genotypes for drought tolerance on physiological and molecular basis.

Differential display (DD) RT- PCR, has been recently reviewed in both eukaryotes and prokaryotes for gene expression studies. Briefly, after cDNA synthesis using reverse transcriptase and an oligo dT primer that anneals to the 3 poly

A tail of mRNA, subsets of cDNA populations for comparison are PCR amplified with short, non-specific oligo nucleotide primers, in combination with oligo dT primers, and visualized on polyacrylamide gels. DD-RT-PCR was first used to isolate plant genes. DD-RT-PCR identified an *Arabidopsis* gene, *ERD15,* which was induced by drought, further demonstrating the activation of common biochemical pathways in response to different stimuli (Paul *et al.,* 2000). In the present study we discuss how DDRT-PCR, genomic and bioinformatics approaches are combined to meet the challenge of identifying and characterizing transcriptional changes during water stress condition.

Analysis of physiological parameters

The ten black peppers genotypes were subjected to water stress, all the genotypes showed different responses to physiological parameters such as relative water content, membrane integrity and days taken for wilting. Relative water content and membrane integrity were reduced with increasing stress intensity. Relative water content was positively correlated with membrane integrity and negatively correlated with stress intensity. The Angamaly (PRS-64) showed highest relative water content, membrane integrity which was on par with Panniyur-5 (PRS-115), PRS-160, Panniyu-8 (PRS-153). The lowest relative content, membrane integrity observed in Panniyur-1(PRS-44) which was par on Karimunda. The number of days taken for wilting varies from one another. PRS-64 takes 23 days to wilt which was at par with Panniyur-8. Panniyur-1 takes 13 days which was at par with PRS-156.

Water stress was imposed on eight months old rooted cuttings of black pepper of fourty cultivars. Among them only six cultivars *viz.*, Kalluvally-4, Kumbakodi, Padarpan, Panniyur-5, Poonjarmunda and Uthirankotta-2 were survived even after the fourth day of water stress. These cultivars showed highest RWC and membrane stability index (Vasantha *et al.,* 2004). Significant differences in relative water content in tolerant and susceptible genotypes of wheat have also been reported (Kraus

et al., 1995). Sibel Tas and Briol Tas (2007) assessed drought stress in terms of relative water content and Membrane integrity. The plant material used in their experiment were hexaploids (*Triticum aestivum*) Atya-85 (drought susceptible) Bezostaja-1 (drought tolerance) and two tetraploids Gerek-75 and Cakmak-79. Significant reductions were observed in RWC and membrane integrity of all cultivars. Of these Cakmak-79 showed the highest membrane integrity.

Relative water content decreased and membrane leakage increased due to water stress in different black pepper genotypes. The RWC in black pepper accessions were reduced after stress induction which ranges from 4.7 (acc 1466) to12% (acc 1411) after 4 days of stress and from 16% (acc 1466) to 46.3% (acc 1411) after 8 days of stress. The membrane leakage increases from 35% (acc 1368) to 83.4% (ace 1409) after 4 days of stress and from 105% (acc 1368) to 253.5% (acc 1411) after 8 days of stress. Acc 1466 and 1368 maintained both lesser reduction in RWC and lesser increase in membrane leakage compared to the other accessions. (Krishnamurthy *et al.,* 2000)

Identification and Characterization of genes operating in stress signaling pathways

In the present study genes operating in ABA dependent signaling pathway, primary carbon metabolism pathway, stress signaling pathway, molecular cheparons, hypothetical and putative proteins were identified by sequence analysis. The results obtained are discussed below

Clone 1

The sequence analysis of clone no 1 which was 859 bp was differentially expressed in water stressed plants. Annotation of sequence by the BLASTn analysis showed homology to NADH dehydrogenase subunit F gene (*Piper nigrum*) which gave 100% identity with 83% of query coverage. Annotation of sequence by the

BLASTx analysis showed homology to NADH dehyrogenase subunit F gene (*Piper nigrum*) 100% identity with 98% query coverage.

NADH dehydrogenase or complex I (EC 1.6.99.3) is a flavoprotein that contains iron-sulfur centers. It catalyzes the transfer of electrons from NADH to coenzyme Q10 (CoQ10) and, in eukaryotes, it is located in the inner mitochondrial membrane. It is one of the "entry enzymes" of oxidative phosphorylation in the mitochondria. (Hugo gene nomenclature committee)

The production of reactive oxygen species (ROS), such as O_2 and H_2O_2, is an unavoidable consequence of aerobic metabolism. In plant cells the mitochondrial electron transport chain (ETC) is a major site of ROS production. In addition to complexes I-IV, the plant mitochondrial ETC contains a non-proton-pumping alternative oxidase as well as two roteonone-insensitive, non-proton- pumping (NADPH) dehydrogenases on each side of the inner membrane: NDex on the outer surface and NDin on the inner surface. Because of their dependence on Ca^{2+}, the two NDex may be active only when the plant cell is stressed. Complex I is the main enzyme oxidizing NADH under normal conditions and is also a major site of ROS production, together with complex III.

The NDin (NADH) and cytochrome oxidase function to limit mitochondrial ROS production by keeping the ETC relatively oxidized. Several enzymes are found in the matrix that, together with small antioxidants such as glutathione, helps remove ROS. The antioxidants are kept in reduced state by matrix NADPH produced by NADP-isocitrate dehydrosinase and non-proton-pumping transhydrogenase activities. When these defenses are overwhelmed, as occurs during both biotic and abiotic stress, the mitochondria are damaged by oxidative stress (Moller, 2001).

Clone 2

The sequence analysis of clone no 2 which was 436 bp was differentially expressed in water stressed plants. Annotation of sequence by the BLASTn analysis showed homology to *Vicia faba* calcium-dependent protein kinasel (CPK1) mRNA complete cds which gave 100% identity with 83% of query coverage. Annotation of sequence by the BLASTx analysis showed homology to calcium-dependent protein kinasel (*Vicia faba*) 100% identity with 36 % query coverage.

Calcium-dependent protein kinases (CPKs) are plant proteins that directly bind calcium ions before phosphorylating substrates involved in metabolism, osmosis, hormone response and stress signaling pathways. CPKs are a large multigene family of proteins that are present in all plants.

Calcium (Ca2+) signaling is a highly integrated signaling network that plays a fundamental role in growth, development and stress responses in plants. Cytosolic Ca2+ concentrations change in complex spatio-temporal patterns in response to various stimuli. In plants, these altered Ca2+ signatures lead to specific cellular responses including stomatal movement, increased water retention, microbial detection and tip structure movement (DeFalco *et al.* 2010; Hashimoto and Kudla 2011).

Clone 3

The sequence analysis of clone no 3 which was 539 bp was differentially expressed in water stressed plants. Annotation of sequence by the BLASTn analysis showed homology to *Piper nigrum* 5.8 ribosomal RNA genes, partial sequence: internal transcribed spacer 2 which gave 100% identity with 83% of query coverage. Annotation of sequence by the BLASTx analysis showed homology to Hypothetical protein BVRB 017900 (*Beta vulgaris subsp. vulgaris*) 41% identity with 86% of query coverage.

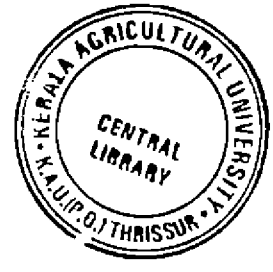
Clone 4

The sequence analysis of clone no 4 which was 649 bp was up regulated in water stressed plants. Annotation of sequence by the BLASTx analysis showed homology to hypothetical protein OsI 31800 (*Oryza sativa indica* Group) 80% identity with 61 % of query coverage. Annotation of sequence by the BLASTx analysis showed homology to hypothetical protein OsI 31800 (*Oryza sativa indica* Group) 80% identity with 61% query coverage.

Clone 5

The sequence analysis of clone no 5 which was 706 bp was differentially expressed in water stressed plants. Annotation of sequence by the BLASTn analysis showed homology to *Vitis vinifera* copper amine oxidasel, transcript variant X2 which gave 81% identity with 36% of query coverage. Annotation of sequence by the BLASTx analysis showed homology to unknown (*Zea mays*) 88% identity with 48% of query coverage.

Copper containing amine oxidizes act as a disulphide-linked homodimer. They catalyze the oxidation of primary amines to aldehydes, with the subsequent release of ammonia and hydrogen peroxide, which requires one copper ion per subunit and topoquinone as a co-factor. These are found in bacteria, fungi, plants and animals. In prokaryotes, the enzyme enables various amine substrates to be used as sources of carbon and nitrogen. In eukaryotes they have a broader range of functions, including cell differentiation and growth, wound healing, detoxification and cell signaling (Kumar *et al.*, 1996).



Summary

6. SUMMARY

Drought is one of the inherent abiotic constraints that affect agricultural productivity worldwide. It is estimated that drought stress can potentially reduce nearly twenty per cent of crop yield around the world. The period (October-May) is considered as water stress period and 20-30 % decrease in yield of black pepper was reported. PRS, Panniyur maintaining germplasm accessions of black pepper under field conditions. Screening and evaluation of genotypes were carried out to identify genotypes showing tolerance to water stress, under field conditions. Based on the phenotypic parameters 15 genotypes were identified. These genotypes were characterized using morphological characters at PRS, Panniyur. Among them ten genotypes were taken for the present study to characterize the genotypes for drought tolerance on phenotypic and molecular basis.

Relative water content in all the ten black pepper genotypes decreased significantly from 5th day to 15th day of stress induction. The maximum relative water content was observed in Angamaly (31.5%) followed by PRS-149 (26.7%) and PRS-155 (26.4%). The minimum relative water content was observed in Karimunda (12.1%) which was on par with Panniyur-1 (12.5%) by the end of 15 days after stress induction. RWC was positively correlated with MI and days taken for wilting whereas it was negatively correlated with stress intensity.

The sequence analysis of clone no 1 which was 859 bp was differentially expressed in water stressed plants. Annotation of sequence showed homology to NADH dehydrogenase subunit F gene (*Piper nigrum*). NADH dehydrogenase or complex I (EC 1.6.99.3) is a flavoprotein that contains iron-sulfur centres. It catalyzes the transfer of electrons from NADH to coenzyme Q10 (CoQ10) and, in eukaryotes, it is located in the inner mitochondrial membrane.

The sequence analysis of clone no 2 which was 436 bp was differentially expressed in water stressed plants. Annotation of sequence showed homology to *Vicia faba* calcium-dependent protein kinase1 (CPK1) mRNA complete cds. These are involved in signaling cascades and in transcriptional control.

The sequence analysis of clone no 3 which was 539 bp was differentially expressed in water stressed plants. Annotation of sequence by the BLASTn analysis showed homology to *Piper nigrum* 5.8 ribosomal RNA genes, partial sequence: internal transcribed spacer 2. Annotation of sequence by the BLASTx analysis showed homology to Hypothetical protein BVRB 017900 (*Beta vulgaris subsp. vulgaris*).

The sequence analysis of clone no 4 which was 649 bp was up regulated in water stressed plants. Annotation of sequence by the BLASTx analysis showed homology to hypothetical protein OsI 31800 (*Oryza sativa indica* Group).

The sequence analysis of clone no 5 which was 706 bp was differentially expressed in water stressed plants. Annotation of sequence by the BLASTn analysis showed homology to *Vitis vinifera* copper amine oxidase1, transcript variant X2. Annotation of sequence by the BLASTx analysis showed homology to unknown (*Zea mays*). In eukaryotes they have a broader range of functions, including cell differentiation and growth, wound healing, detoxification and cell signaling.

The sequence from differentially expressed TDFs showed homology to copper containing amine oxidases which have a broader range of functions, including cell differentiation and growth, wound healing, detoxification and cell signaling. It contributes to terminal polyamines oxidation in peroxisomes. Polyamines are involved in different physiological processes such as growth development and response to abiotic stress. The other major genes identified were NADH dehydrogenase, heat shock proteins, ribosomal RNA gene, protein kinase which is all operating in major signal transduction pathways.

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APPENDIX I

Composition of different media used in the study

1. **Luria Bertoni (LB) broth**

Tryptone	10g
Yeast Extract	5g
Nacl	5g
pH adjusted to	7+- 0.2

Distilled water Make up to 1000ml

2. **Luria Bertoni agar medium**

Tryptone	10g
Yeast Extract	5g
Nacl	5g
Agar	20g
pH adjusted to	7+- 0.2

Distilled water Make up to 1000ml

APPENDIX II

Buffers and dyes used in gel electrophoresis

1.6x Loading / tracking dye

Bromophenol blue	0.25%
Xylene cyanol	0.25%
Glycerol	30%

The dye was prepared and kept in fridge at 4^0C

2. Ethidium bromide (interlating dye)

   The dye was prepared as a stock solution of 10mg/ml in water and was stc

   at room temperature in a dark bottle

3. 50x TAE buffer (pH 8.0)

Tris base	242.0g
Glacial acetic acid	57.1
0.5M EDTA (pH 8.0)	100ml
Distilled water	1000ml

   The solution was prepared and stored at room temperature

4. 10X MOPS buffer (pH 7)

200mM MOPS (pH 7)	41.85gm
80mM Sodium acetate	6.56gm
10mM EDTA	3.725
DEPC water	1000ml

The solution was prepared in DEPC autoclaved water and stored at room tempera

APPENDIX III

Primers used for DD-RT-PCR

Primer No.	Sequential information(5' to 3')	No. of bases
T11C	AAGCTTTTTTTTTTC	16
AP1	AAGCTTGATTGCC	13
AP2	AAGCTTCGACTGT	13
AP3	AAGCTTTGGTCAG	13
AP4	AAGCTTCTCAACG	13
AP5	AAGCTTATAGGC	13
AP6	AAGCTTGCACCAT	13
AP7	AAGCTTAACGAGG	13
AP8	AAGCTTTTACCGC	13

APPENDIX 1V

Composition of the reaction mixture for colony PCR

Component	**Volume/reaction**
10X tac buffer	2 µl
dNTP(10mM)	2 µl
$Mgcl_2$	1.2 µl
pGEMT forward sequencing primer(10 µM)	0.4 µl
pGEMT reverse sequencing primer(10 µM)	0.4 µl
Tac polymerase (5u/µl)	0.1 µl
Water(Nuclease free)	13.9 µl
Total	20 µl

PCR cycle set for colony PCR

S.No	Step	Temperature	Time
1	Initial denaturation	95^0 C	3 min
2	Denaturation	94^0C	30s
3	Annealing	60^0C	30s
4	Extension	72^0C	1 min
5	Step 2 to 4	30cycles	
6	Final Extension	72^0C	10 min
7	Hold	4^0C	10 min

MOLECULAR CHARACTERIZATION OF BLACK PEPPER (*Piper nigrum* L.) GENOTYPES FOR DROUGHT TOLERANCE

By

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ABSTRACT OF THE THESIS

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Abstract

Black pepper (*Piper nigrum* L) the "King of spices" and a perennial climber belonging to the family Piperaceae, is known for its quality and it fetches premium price in the international market. Ninety per cent of the area under black pepper is rainfed. As India is the primary centre of diversity of black pepper, the indigenous genetic resources are reservoirs of useful genes for plant improvement programmes. Black pepper is grown mainly in Kerala, where water deficit during off season *viz.*, December-May is a common feature. To avoid reduction in yield during water stress condition, cultivation of drought tolerant varieties is essential (Rajagopal and Balasimha, 1994).The present study on "Molecular characterization of black pepper (*Piper nigrum* L.) genotypes for drought tolerance" was under taken at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period 2013-2015 with the objective of screening the selected black pepper genotypes for drought tolerance and identifying differentially expressed, up regulated, down regulated genes by transcriptome analysis.

The genotypes PRS 64(Angamaly), PRS 155(wild type), PRS 115(Panniyur-5), PRS 149(wild type), PRS 156, PRS 160(Hybrid), PRS 161(Hybrid), PRS 153(Panniyur-8), PRS 44(Panniyur-1) and Karimunda were selected for the study. Water stress was imposed on all the genotypes for 15 days continuously on six month old rooted cuttings and observations on relative water content and membrane integrity were taken at 5 days interval. Days taken for wilting were also noted.

The genotypes showed different responses to physiological parameters such as relative water content, membrane integrity and days taken for wilting. Relative water content and membrane integrity were reduced with increasing stress intensity. Relative water content was positively correlated with membrane integrity and negatively correlated with stress intensity. The PRS-64(Angamaly) showed highest relative water content and membrane integrity which was on par with PRS-

115(Panniyur-5), PRS-160, PRS-153(Panniyur-8). The lowest relative content a membrane integrity was observed in PRS-44(Panniyur-1) which was on par wi Karimunda. The number of days taken for wilting varies between genotype Angamaly took 23 days to wilt which was on par with Panniyur-8. Panniyur-1 to 13 days to wilt which was on par with PRS-156. PRS-64 identified as most droug tolerant genotype and PRS-44 identified as susceptible genotype

The mechanisms for abiotic stress tolerance are based on activation a regulation of specific set of stress-related genes which are involved in signallir transcriptional control, and protection of membrane proteins. The technique used analyse the transcriptome was differential display-RTPCR which allows extensi analysis of gene expression among several cell populations (Sturtevant, 2000). T gene expression analysis requires good quality RNA which was isolated by Tri reagent method with utmost care to prevent its degradation by nucleases.

One µg of total RNA from the two treatments were taken for DD-RT-P analysis. The first strand cDNA was synthesized from the above RNA samples usi HT11G (AAGCTTTTTTTTTTG). Each first strand cDNA was used for the seco amplification with 8 different arbitrary primers. The PCR product was resolved ir per cent denaturing urea polyacrylamide gels and visualized after silver staining. T gel was dried overnight for the elution and further analysis. The up regulated, do regulated and differentially expressed cDNA fragments were retrieved from the and reamplified with the same set of primers as in the initial DD-RT-PCR reacti and analysed electrophoretically. The agarose gel electrophoresis showed that transcript derived fragments (TDFs) obtained were relatively short (400-900b TDFs were cloned using pGEMT vector. The clones were sequenced at Sci Genor Cochin. The sequence data obtained were analysed by in silico tools. The sequer from differentially expressed TDFs showed homology to copper containing am oxidases which have a broader range of functions, including cell differentiation a

growth, wound healing, detoxification and cell signalling. It contributes to terminal polyamines oxidation in peroxisomes. Polyamines are involved in different physiological processes such as growth development and response to abiotic stress. The other major genes identified were NADH dehydrogenase, heat shock proteins, ribosomal RNA gene, protein kinase which is all operating in major signal transduction pathways.