World Research Journal of
PHARMA TECHNOLOGY
ISSN: 2454-5546
Research Article

RESPONSE TO SALINITY: A STUDY ON BLACK CUMIN (*Nigella sativa L.*)

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ABSTRACT

Abiotic stress such as salinity, drought, cold or high temperature influences crop productivity to a great extent. Several genes are responsible for the mechanism of salinity tolerance and biosysnthesis of osmotically compatible solutes in cytosol to cope with salt stress. Germination percentage along with several growth parameters like root and shoot length and fresh weight gain of *Nigella sativa* were studied under different salinity conditions. Gradual increase in salinity adversely affected the rate of germination and growth of the seedlings, coupled with gradual decreases in the root and shoot length. The potency of black cumin to fight against salinity was further studied by estimation and profiling of total buffer soluble proteins which were evidently increasing with enhanced salinity. However, phenolics and total flavonoid contents were measured to be highest less than 200 mM NaCl treatment followed by an immediate drop at 50 mM NaCl. The same trend was followed by the estimated figures of DPPH radical scavenging assay, profiling and densitometric scan of peroxidase and acid phosphatase, the most frequently used markers of abiotic stress. Such trend of the studied parameters can be interpreted by the probable sudden shock under 50 mM salinity. But, further enhancement of competence at 200 mM salinity points to the probable induction of the antioxidant activity and ROS scavenging machinery in *Nigella sativa* that might help the plant to withstand further salinity stress. The present work may recommend *Nigella sativa* as a worthy crop to grow in saline, but yet unused coastal lands of our country at least for trial basis.

Keywords: *Black cumin, Nigella sativa L., Pharmacognosy, Botany.*

INTRODUCTION

Abiotic stress such as salinity, drought etc. are serious threats for medicinal and field crops all over the world, especially in arid and semi-arid regions. Germination is the first sensitive stage in the life cycle of a plant and establishment of seedling is highly affected under stress. It has been estimated that loss of more than half of the yield of some major crop plants can be attributed to the unfavorable environments like drought or high salinity (Cortina & Culiáñez-Macià, 2005). A handful of reports which are available on the cultivation of medicinal plants in saline habitat include effect of salinity stress on germination and seedling growth of *Nigella sativa* (Razmjoo *et al*, 2008), *Helianthus annus* (Khan *et al,* 1994; Daniella *et al,* 2004; Mutlu & Bozcuk, 2007), *Glycine max* (Essa, 2002), *Plantago spp.,* (Mackova *et al,* 1988) and some other medicinal plants (Ibrar & Hussain, 2003; Hanselin & Eggen, 2005).

Soil salinity is a major problem in agriculture since majority of crop plants is sensitive to salt stress. In nature, sodium chloride is the most common factor causing salt stress upon plant metabolism (Veneti, 2005). It affects plants in three major ways, *i.e.,* reduces water potential, and causes cellular toxicity and ion imbalance in cell. Salt tolerance is considered to be a complex multigenic character (Shannon, 1996 and flowers, 2004) expressed to produce several enzymatic and non-enzymatic antioxidants. Antioxidants are molecules capable of inhibiting the oxidation of other molecules. These are often reducing agents such as thiols, ascorbic acid or polyphenols

(Sies Helmut, 1997). Hence, antioxidants are effective markers for stress tolerance for any plant species under certain biotic or abiotic stress.

Under physiological steady-state conditions, there is a balance between the productions and scavenging of ROSs (Skopelitis *et al*., 2006). But this cellular steady state condition can be disturbed by a number of certain adverse environmental factors (*e.g*. Heat stress, Salinity stress etc) leading to oxidative stress. Exposure of plants to these abiotic stress results in the high production of reactive oxygen species (ROS) as by products, which damage the cellular components (Noctor and Foyer, 1998). Superoxides generated due to salt stress unbalances the cellular redox system in favour of oxidized forms resulting in oxidized damage to lipids, proteins and nucleic acid (Halliwell and Gutteridge, 1989). Plants have developed several enzymatic and non-enzymatic systems to counteract these ROSs, and protect cells from oxidative damage (Sairam and Tyagi, 2004).

Over the past decade evidences have been accumulated that plant polyphenols and especially flavonoids are the most important class of defensive non-enzymatic antioxidants. Flavonoids comprise the major groups of soluble phenolics in *Nigella sativa L*. Flavonoids confer UV-protection, signal molecules, phytoalexins.

Phenolics are traditionally considered as defensive compound that protects the plant from free radicals. The hydroxyl groups present in these compounds enable them to act as free radical scavenger, thus, up-regulating antioxidant defence mechanisms in plant tissues (Hatano *et al*., 1989; Caturla *et al,* 2003). Polyphenolic compounds are also reported to have inhibitory effects on mutagenesis and carcinogenesis in human beings (Gülçin *et al.*, 2003).

Every plant has an original and unique flavonoid profile, which makes quantification difficult. For this reason, the method frequently used to determine the total flavonoid content of herbal materials includes hydrolysis of the glucosides to reduce the variety and number of analysis. DPPH has often been used as a free radical producer to evaluate reducing substances (Cotelle et al., 1996) and is a useful reagent for investigating the free radical scavenging activities of polyphenol compounds (Duanet et al., 2006). DPPH can only be dissolved in organic media, especially in ethanol, thus puts up an important constraint while interpreting the role of hydrophilic antioxidants. However, antioxidant potency and polyphenol content of grape cultivars varying in their skin colours were earlier reported by many researchers following DPPH radical scavenging method (Pakhale *et al.*, 2007, Montealegre *et al.*, 2006). Later, antioxidant activity and phenolic composition of organic and conventional grapes and wines were extensively studied by Mullero *et al.* (2010).

Isozymes are induced to scavenge the reactive oxygen species and thus are effective markers of stress. Peroxidases (EC 1.11.1.7) are important ROS scavengers that either bound to cell wall or sequester in the protoplast (Madder, 1976). Peroxidases are widely found in animals, plants and microbes and oxidize a vast array of compounds (electron donors) in the presence of hydrogen peroxide (H_2O_2). Acid phosphatase is a group of important enzymes that catalyzes the hydrolysis of a faction of phosphomonoester and phosphoproteins but not the phosphodiester bond. Under phosphorus deficiency due to abiotic stress, the enzyme is induced to maintain certain level of inorganic phosphate inside the cell (Olmos and Hellin, 1997)**.** Acid phosphatises are mainly localised in the cytoplasm, vacuole and cell wall. Protective roles of the antioxidant enzymes in temperature and salt stress have been reported from a

number of plants (Almeselmani *et al*., 2006; Jaleel *et al*., 2007; Esfandiari *et al.*, 2007).

Nigella sativa L. is the "miracle herb", which can be effectively used for treatment of various stomach problems, cough and cold and even asthma. The optimum amount of thymoquinone, carvacrol, anethol and terpenol in the seed oil offers therapeutic property against hypertension, renal disorder and skin diseases. It is one of the widely cultivated crops that grow well in acidic, alkaline as well as neutral soil types and used as an inevitable spice in our cuisine. The present study focused on the germination and different growth parameters of black cumin up to 200 mM NaCl treatment as well as the expression of effective antioxidants to withstand salt stress.

MATERIALS AND METHODS

Seeds of *Nigella sativa* were treated with three different NaCl gradients *i.e.* control, 50mM and 200mM NaCl. At the outset all the seeds were germinated at control condition, of which two sets were exposed to 50 mM NaCl treatment after three days of germination. One of them was further treated with 200 mM NaCl again after three days and finally ten days old seedlings were harvested from the three different sets for the experiment.

Root /shoot length and Fresh weight: Ten days old seedlings were measured for their shoot and root length and weighed for fresh weight. An average of data obtained from fifteen seedlings were represented from each set of treatment.

Genomic DNA Isolation: Total genomic DNA pool was indeed not a pointer of antioxidation potency, but was isolated, estimated and visualized from both the materials for documentation. Genomic DNA was isolated following CTAB method and the isolated DNA was checked for its purity and quantified using an UV spectrophotometer (UV-1800, Shimadzu). The DNA pool was visualised by 2% agarose gel electrophoresis using ethidium bromide as the intercalating fluorescent dye.

Estimation of Total Buffer Soluble Protein: the seedlings were crushed and were extracted at 4^0C in the protein extraction buffer (pH 6.8). The extract was centrifuged at 15000 rpm for 15 min at 4^0C and the supernatant was used for the spectrophotometric estimation of total protein at 660 nm (Lowry *et al*., 1951). Bovine serum albumin was used as the standard.

1D Protein Profiling by SDS-PAGE: 1D SDS Polyacrylamide Gel Electrophoresis was performed to resolve the protein pool from fruit extracts. Detergents like SDS, DTT and β-marcaptoethanol were used in the buffers to cleave the di-sulphide bonds of the folded protein molecules into shorter polypeptides. Overnight exposure to Coomassie Brilliant Blue G was used to stain the polypeptide bands across the lanes.

DPPH Radical Scavenging Assay: The free radical scavenging activity of different plant extracts were determined using the stable radical DPPH (2, 2-diphenyl1-1picrylhydrazyl) following Blois (1958).

Isozyme Profiling: Native gel electrophoresis plant extracts was performed separately to obtain the isoforms of Peroxidases and Acid Phosphatases. Substrate specific stains were used to visualize the bands against the detergent free polyacrylamide gel. In case of POX, H_2O_2 coupled with orthodynacidine, and for Acid Phosphatase, α-napthyl acid phosphate with Fast Blue BB salt imparted dark brown stain of isoforms of the respective isoenzymes. Densitometric scan of the bands was done by a Gel Documentation System (Gel Doc EZ, Biorad) using the Image Pro software. The position and intensity of each isoform was documented and graphically represented as absorbance peaks.

Estimation of Flavonoids and Total Phenolic Compounds: the plant sample extracts were prepared in methanol and centrifuged at 1000rpm for 15 min at 4^0C. The supernatant was divided into two portions. Flavonoid compounds were spectrophotometrically estimated from one fraction at 765 nm using quercetin as standard (Chang *et al*., 1977). The other portion was used to estimate total phenolic compounds at 510 nm using gallic acid as standard (Malick & Singh, 1980).

RESULT AND DISCUSSION

Root /Shoot length and Fresh weight:

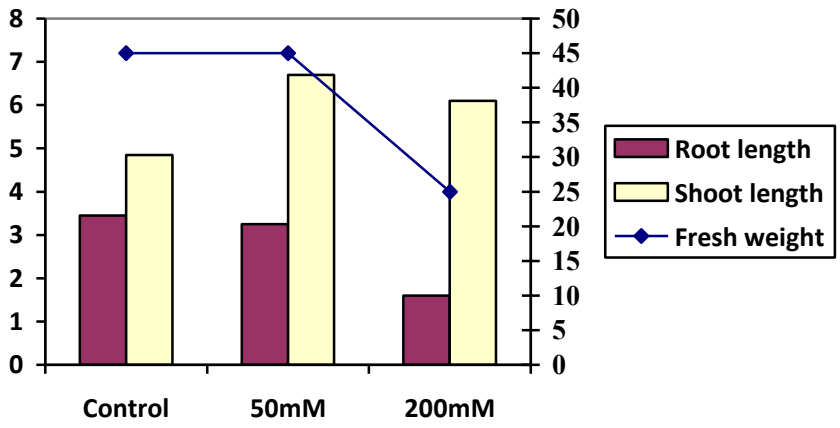


Fig.1: Root/Shoot length and Fresh weight

The fresh weight of control and 50mM concentration was found to be equal (Fig.1), whereas the fresh weight of 200mM treated seedlings was found to be decreased.

Fig.2.a) Control b) 50mM NaCl c) 200mM

The average root length and shoot length of control set (Fig.2a) was- 3.45cm and 4.85cm respectively. The root lengths of 50mM and 200mM treated seedlings (Fig 2b,c) were found to be decreased (3.25 and 1.6 cm respectively) after ten days of treatment (Fig.1) whereas the shoot length of both (Fig.2a,b) enhanced (6.7cm in 50 mM and 6.1 cm in 200mM respectively) (Fig.1).

Total Genomic DNA: The isolated genomic DNA (Fig.3) was tested for its purity by measuring the optical densities at 260 and 280 nm (Table 1). The DNA was quantified in assumption that 1OD at 260 nm is equivalent to 50 ng/ml DNA.

Table 1: **O.D. values of genomic DNA pool**

Plant Material	OD (260 nm)	OD (280 nm)	OD_{260}/OD_{280}
Nigella sativa	0.33	0.18	1.87

The ratio indicates that the isolated DNA pool was free from protein contamination and the estimated amount in *Nigella sativa* was 16.5 ng/ml.

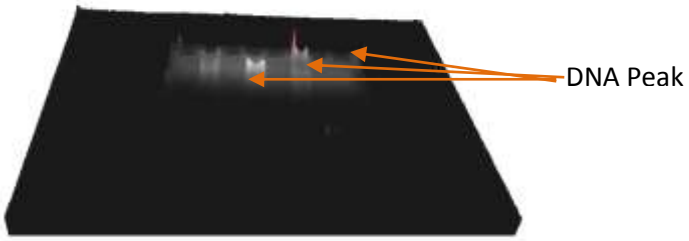


Fig.3. Genomic DNA pool showing the DNA peaks of the plant samples

Total buffer soluble protein: The estimated amount of total protein (Table 2, Fig 4) was higher in the 200mM stress treatment than the control and 50mM sets.

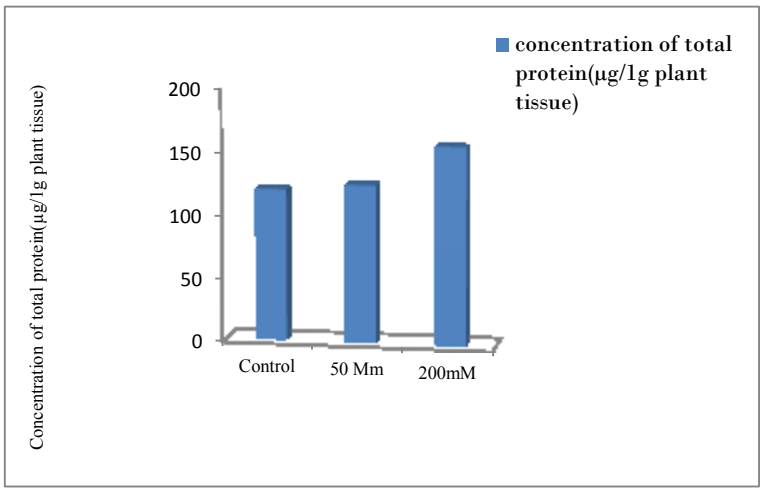


Fig.4. Total buffer soluble protein

Table 2. Concentration of Protein

Material	Protein Conc. (µg/1g)
Control	120
50mM	125
200mM	157

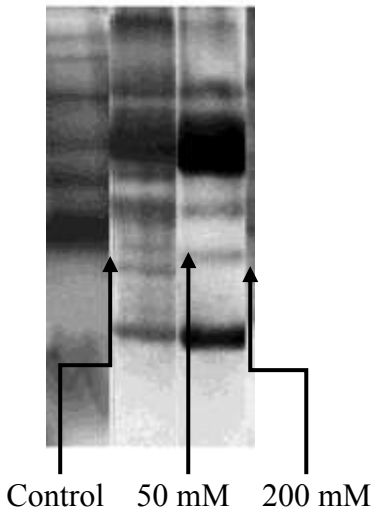
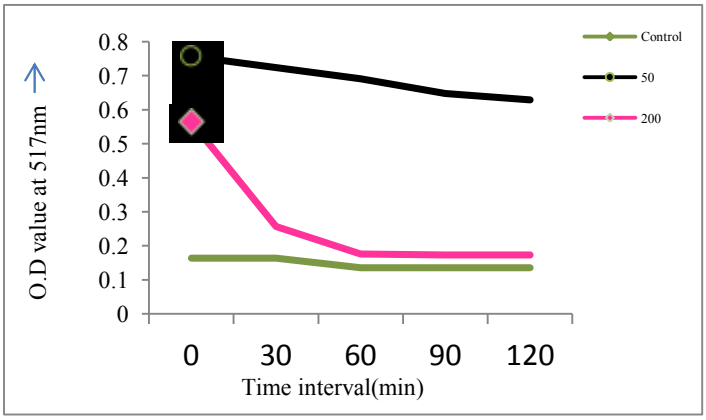


Fig.5. 1D polypeptide profile (SDS-PAGE)

1D Polypeptide Profile (SDS-PAGE)

The 1D polypeptide profile (SDS PAGE) (fig.5), shows that some polypeptide bands are more prominent in 200mM treated seedlings than in the control and 50 mM sets. This implicates the possibility of up-regulation of the respective genes and thus, enhanced induction of these polypeptides under higher salinity. Moreover, absence of certain bands may point to the suppression of certain proteins or down regulation of their respective genes at 200 mM salt treatment. Some of the polypeptide bands present in the control lane is not evident in the stressed condition. This may point to the fact that maybe these polypeptides are suppressed under stress.

DPPH Radical Scavenging Assay



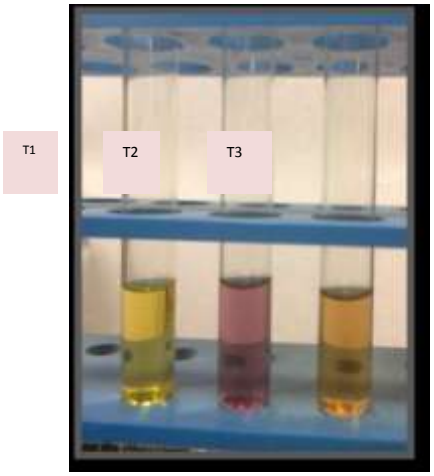


Fig 6 (a), (b): DPPH radical scavenging assay

The radical scavenging assay of the control set followed a constant steady state condition throughout (Fig.6a), whereas the DPPH retained colour in the extract of 50mM set (Fig.6b) even after 120 minutes and a gradual but constant decline was evident (Fig.6a); in contrast the 200 mM treatment enabled the plant to oxidise the free radicals as fast as by 50 min (Fig.6b) and the graph, following a sharp decline reached a steady state thereafter.

Isozyme Profile (Native gel electrophoresis)

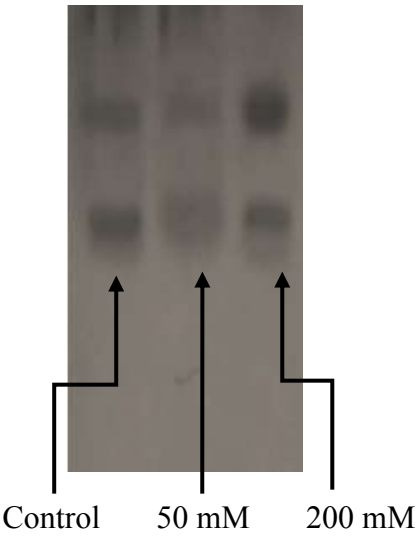


Fig 7: Isoforms of Peroxidase

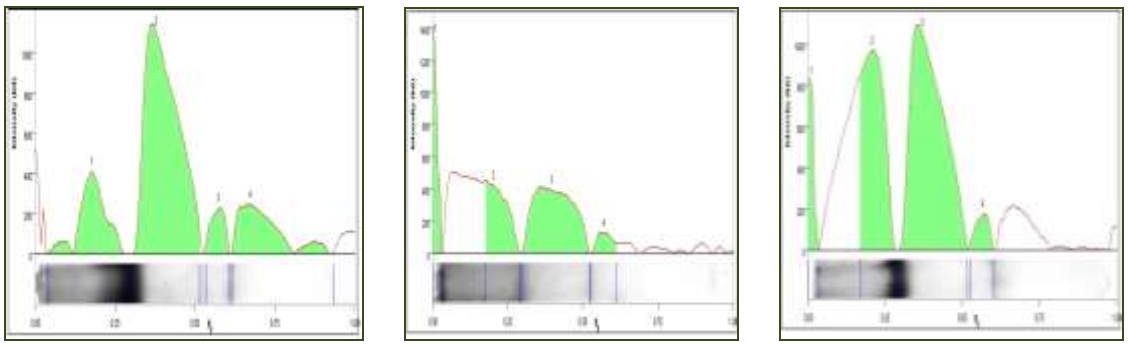


Fig 8: Densitometric scan of peroxidase isoforms

The photograph (Fig.7) shows the isoforms of peroxidases expressed in control, 50mM NaCl and 200mM NaCl treatments respectively. The intensity of bands in 200mM is higher than those of 50mM set (Fig.8) that may indicate enhanced ROS scavenging potential of *Nigella* seedlings under increased stress.

Isozyme Profile (Native gel electrophoresis)

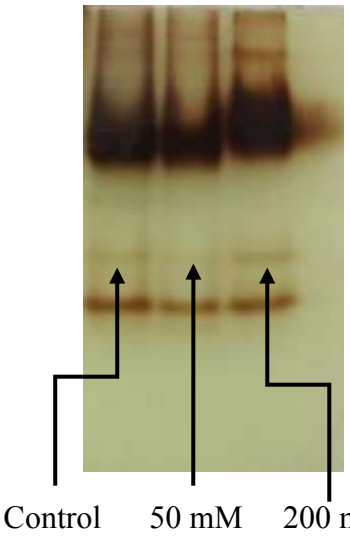


Fig 9: Isoforms of Acid Phosphatase

The photograph (Fig 9) shows the isoforms of acid phosphatase expressed in control, 50mM NaCl and 200mM NaCl treatments respectively. The intensity of bands in 200mM is higher than those of 50mM set (Fig.10) that may indicate enhanced ROS scavenging potential of *Nigella* seedlings under increased stress.

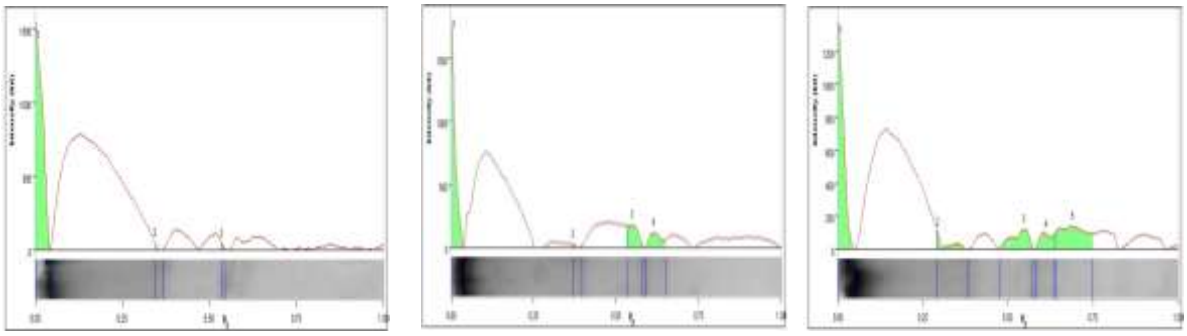


Fig 10: Densitometric scan of acid phosphatase isoforms

The picture (Fig.7 and Fig.9) shows the Isoforms of Control, 50mM NaCl, 200mM NaCl stress respectively. The intensity of bands in 200mM is higher than the 50mM and control. Both the profile pictures (Fig.7, Fig.9) points to the enhanced expression of antioxidant enzymes in 200mM treatment pointing to sudden augmentation of scavenging machinery in increased salinity.

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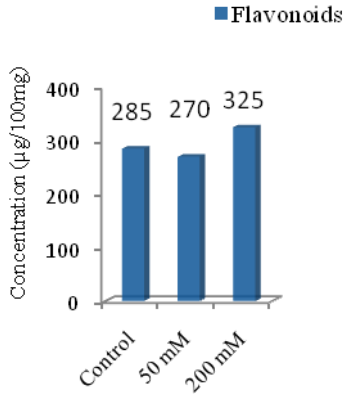
Research Article



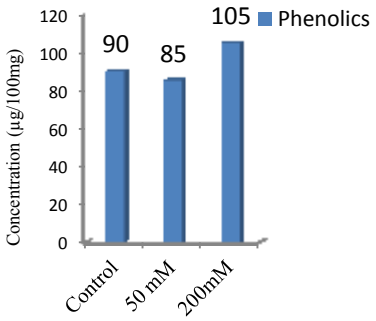


Fig 11: Total Flavonoids

Fig 12: Total Phenolics

The concentration of total flavonoids (Fig 11) and phenolic contents (Fig 12) was found to be at the highest at the 200mM NaCl concentration that supports the previous data.

CONCLUSION

In the above experimental study, it has been found that with the increase in salinity in the soil, the stress produced in the plant has imposed an adverse effect on the germination rate as well as growth parameters in *Nigella sativa*. It has been observed in the experiment that the growth of root and shoot of *Nigella sativa* varied in the salt stress condition as compared to the controlled set of the plant.

However, in enzyme activity analysis, it was found that the antioxidant potency of the plant suddenly decreased in the salt stress condition of 50mM. This situation arised probably due to the sudden shock the plant received in that condition. With further enhancement of salt stress upto 200mM, the plants started to show recovery in order to withstand the stressed condition and therefore, the NaCl treatment of plant upto 200mM shows an enhances competence. This tradition could be seen from the intensity of the bands of the isoforms of peroxidase and acid phosphatase respectively. The isoforms shows the fact that the sudden augmentation of ROS scavenging machinery has helped the plant to withstand the further stress and cope up with situation for its survival. If we further go through the non-enzymatic antioxidant activity in stressed plants, it could be seen that both phenolics and flavonoid concentrations were much higher in salt stressed condition than in controlled set. All these above conditions point to the fact that *Nigella sativa* has the ability to grow in a wide range of soil, from acidic to alkaline.

From the above experiments, it was also observed that the trend of root and shoot lengths under stress condition was in harmony with previous observation of Hazar and Zidan (1996). Further investigation and thorough experiment workout and analysis is required in order to draw any final conclusion and statement.

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