

## Exogenous proline and proline-enriched *Lolium perenne* leaf extract protects against phytotoxic effects of nickel and salinity in *Pisum sativum* by altering polyamine metabolism in leaves

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**Abstract:** Pea plants (*Pisum sativum* L.) were exposed to NaCl and/or NiCl<sub>2</sub> stress to investigate whether pure proline and *Lolium perenne* L. (LP) leaf extract could efficaciously protect against the phytotoxicity generated by nickel and/or salinity stress in pea. Both stress factors (salinity and nickel) significantly inhibited growth, total chlorophyll content, photosynthetic activity, stomatal conductance, intercellular CO<sub>2</sub> level, relative water content (RWC), and membrane stability index (MSI). However, superoxide, hydrogen peroxide, proline, glycine betaine, total free amino acids, total soluble sugars, total phenolic compounds, tocopherol contents, lipid peroxidation, and electrolyte leakage were significantly increased. Similarly, free, soluble-conjugated, and insoluble-bound polyamine contents [spermine (Spm), spermidine (Spd), and putrescine (Put)] and activities of polyamine-biosynthetic enzymes (arginine decarboxylase, ornithine decarboxylase, and S-adenosylmethionine decarboxylase) in leaves were also increased in response to nickel and/or salinity stress. Exogenously applied proline and LP leaf extracts significantly overcame the nickel- and/or salinity-induced toxic effects on growth, RWC, and various photosynthetic attributes. The follow-up treatment with proline and LP leaf extract detoxified the stress caused by NiCl<sub>2</sub> and/or NaCl by suppressing the lipid peroxidation and electrolyte leakage, accelerating the activities of polyamine-biosynthetic enzymes, and improving the MSI, leaf polyamines (Spm, Spd, and Put), and organic osmolytes (free proline, glycine betaine, total free amino acids, total soluble sugars, total phenols, and tocopherol contents). Natural proline (LP leaf extract) proved better than pure proline in improving growth, photosynthetic activity, RWC, organic osmolytes, and polyamine metabolism. Since LP leaf extract is enriched with substantial amounts of proline along with many other essential nutrients, it was as efficacious as pure proline in improving growth, some major physiological attributes, and polyamine metabolism in pea under nickel and/or salinity stress. Thus, it can be used as an alternative inexpensive source of proline for its utilization as a mitigating agent for protecting plants against the phytotoxic effects of nickel and/or salinity stress.

**Key words:** Salt stress, nickel stress, proline, polyamine, photosynthesis, osmolytes

### 1. Introduction

Proline is a proteinogenic amino acid, highly essential for various vital metabolic processes within the plant tissues. It is naturally present in plants, microbes, and animals (Csonka et al., 1988; Csonka and Hanson, 1991; Szabados and Savoure, 2010). It occurs mostly in different plants such as *Carica papaya* L., *Cynodon dactylon* (L.) Pers., *Euphorbia hirta* L., *Psidium guajava* L., and *Melia azedarach* L. (Suresh et al., 2008). Proline was first found in *Lolium perenne* L. (LP) leaf extract (Kemble and

MacPherson, 1954). It promotes the deposition of useable nitrogen and enhances membrane stability under stressed conditions. Like other osmolytes, proline is also naturally produced in plants grown under stressed conditions for osmotic adjustment. It was reported that proline has marked osmoregulatory effects in plant cells under hyperosmotic stress generated by various abiotic factors (drought, salinity, cold, etc.) and that it acts as an efficient osmolyte to prevent damage to cells (Heuer, 1994). High ratios of cytoplasmic osmolytes like proline are thought

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to maintain the cellular water potential at a level lower than that of external water potential; thus, water moves into the cell, stays there, and ensures lower elevations in ionic strength (Heuer, 1994). In addition, accumulation of proline under stressed conditions may not only perform an osmoregulation action, but it could also be involved in the process of providing cells with a pool of the precursors needed to generate other molecules associated with various abiotic stress responses (Sanchez et al., 2008). There are various reports that indicate that stressed conditions trigger the accumulation of proline in plant tissues and are responsible for better growth and productivity (Ali et al., 2008; Yang et al., 2011; Bauddh and Singh, 2012; Hayat et al., 2012; Yusuf et al., 2012; Agami, 2013; Wani et al., 2013). Abiotic stresses drastically affect crop production by reducing yield. Plants face many distinctive abiotic stresses at different stages of their growth and development. Water, light, heat, and salt stress are the major abiotic stresses that ultimately affect plant growth. Among these, salt stress (salinity) has a significant limiting effect on plant growth and productivity (Parida and Das, 2005), and this effect varies from place to place. Plants cope with these abiotic stresses through a variety of mechanisms, including changes in morphological, physiological, and biochemical processes (Shahid et al., 2011; Balal et al., 2012; Shahid et al., 2011, 2014). High concentrations of salts affect physiobiochemical aspects such as photosynthesis rate, transpiration rate, stomatal conductance, water use efficiency, sugars, proteins, enzymatic activities, and water metabolisms (Khan et al., 2009; Omoto et al., 2012; Sandoval-Gil et al., 2012; Wani et al., 2013; Jamoussi et al., 2014). Nutritional imbalances can also create an antagonistic effect between sodium ( $\text{Na}^+$ ) and potassium ( $\text{K}^+$ ) for crucial binding sites in various physiological and cellular metabolic processes (Tester and Davenport, 2003). All these changes in morphophysiological, biochemical, water-related, and ionic attributes limit plant growth and the subsequent yield.

Stress generated by heavy metals such as cadmium, lead, or nickel is also another environmental threat to agricultural productivity and human health (Jarup, 2003; Singh and Pandey, 2011). Among the heavy metals, Ni has been classified as an essential micronutrient (Brown et al., 1987) and is an inherent part of the enzyme urease (Sirko and Brodzik, 2000). This enzyme catalyzes the breakdown of urea into ammonia and  $\text{CO}_2$ . It is also linked with a few other metalloenzymes that regulate various plant processes (Giridhara and Siddaramappa, 2002). However, plants need Ni in very low amounts (below  $1.7 \mu\text{mol kg}^{-1}$  Ni) (Dalton et al., 1988). Excessive application of phosphate fertilization and pesticides, untreated sewage water, and effluents from the metal and battery industries are important agents that elevate Ni concentrations in soil

and environment. Supraoptimal ratios of Ni induce highly deleterious effects on germination (Khan and Khan, 2010), green pigments (Drazkiewicz and Baszynski, 2010), and photosynthesis (Velikova et al., 2011). On the other hand, Ni alters the plant moisture status (Pandey and Sharma, 2002) and augments the antioxidant activities (Yusuf et al., 2012).

Polyamines are ubiquitous compounds with low molecular weight and are present in all living organisms (Smith, 1985). They are actively involved in various kinds of metabolic and physiological processes such as DNA regulation, gene transcription, morphogenesis, embryogenesis, cell differentiation, and fruit ripening (Liu and Moriguchi, 2007). In addition to this, they are also involved in responses to various stresses like salinity, temperature, and ozone. (Liu et al., 2007). The most commonly found polyamines are spermine (Spm), spermidine (Spd), and putrescine (Put), which may be present in 3 forms (free, bounded, and conjugated). Various investigations on *Oryza sativa* L., *Lycopersicon esculentum* L., *Triticum aestivum* L., and *Helianthus annuus* L. showed marked alterations in polyamine contents and their biosynthesis under stressed environments (Krishnamurthy and Bhagwat, 1989; Botella et al., 2000; Li and Chen, 2000; Alcazar et al., 2006). Since polyamine contents vary under stressed conditions, they are implicated as indicators of stress tolerance in the plant genome. Stressful conditions accelerate the accumulation of endogenous polyamine contents in plant tissues, which results in scavenging of the free radicals of oxygen, stimulation of ATP-formation, and improvement in membrane stability (Bouchereau et al., 1999).

Vegetables are an excellent source of phytonutrients and phytochemicals, which are essential for various metabolic activities in the human body (Juge et al., 2007; Dinkova-Kostova, 2008; Noreen and Ashraf, 2009). Peas are an excellent human food; they contain carbohydrates, proteins (Hussein et al., 2006), fats, minerals, vitamins (A, B, and C) in reasonable amounts (Choudhary, 1990), and water-soluble fiber and antioxidants (Noreen et al., 2009). Pea is the fourth most important grain legume crop of the world, as measured by production ( $441.53 \times 10^3$  t). Extensive investigation has been done to evaluate drastic stress-induced effects on the morphophysiological and biochemical attributes of pea (Guerrier and Patolia, 1989; Hernandez et al., 1995; Najafi et al., 2007; Noreen and Ashraf, 2009; Shahid et al., 2012, 2013). However, information on the mitigation of salinity- and/or nickel-induced stress by exogenously applied proline in pea is lacking. Like in various other naturally proline-accumulating plants (Suresh et al., 2008), LP leaves also contain high concentrations of proline (Kemble and MacPherson, 1954). Beside this, LP leaf extract contains

significant amounts of various inorganic nutrients and organic compounds like nitrogen, phosphorus, calcium, potassium, magnesium, fiber, carbohydrates, and proteins (Louahlia et al., 1999; Harrington et al., 2006). However, utilization of this natural proline source in mitigating salinity- and/or nickel-induced deleterious effects cannot be found in the literature.

We therefore hypothesized that the exogenous application of pure proline and proline-enriched LP leaf extract as a foliar spray could effectively diminish salinity- and/or nickel-induced toxic effects on the growth of pea by altering polyamine metabolism and organic osmolytes in leaves. Thus, the current study was organized with 2 main objectives: to examine whether or not foliar application of proline could protect pea against salinity and/or nickel stress, and to investigate how efficacious the LP leaf extract is in regulating growth, selected major physiological processes, organic osmolytes, and the polyamine metabolism associated with salinity and/or nickel tolerance of pea in comparison to the pure proline.

## 2. Materials and methods

The seeds of *Pisum sativum* L. 'Sprinter' were obtained from the Ayyub Agriculture Research Institute, Faisalabad, to investigate the plausible role of exogenously applied proline and LP leaf extract in enhancing the growth of pea plants exposed to heavy metal (Ni) and salinity stress (NaCl). The seeds were sown in plastic pots (10-L capacity; perforated bottom) filled with Astatula fine sand (hyperthermic, uncoated Typic Quartzipsamments), and moistened with deionized water as a growth medium. The seeds were disinfected with a 10% sodium hypochlorite solution before sowing. The experiment was repeated 5 times and there were 5 pots per replication. Ten seeds per pot were sown; following the emergence of the first true leaves (10 days after germination), the number of plants per pot was adjusted to 6 by thinning out the weak and less vigorous plants. Under each replication, data were recorded from each pot and then the average was calculated. The plants were irrigated with deionized water and half-strength Hoagland solution on alternate days. Overall, approximately 300 mL of deionized water with Hoagland solution (30 mL/L) was applied to each pot to fulfill their irrigational requirements. The pots were placed in a growth chamber adjusted to 25/18 °C day/night, relative humidity of 75%/85% (day/night), and light intensity 62,200 lx from fluorescent tubes. After 20 days of sowing, the seedlings were treated with NaCl (100 mM) and/or NiCl<sub>2</sub> (100 µM) along with the nutrient solution. Ten days after NaCl and/or NiCl<sub>2</sub> application, plants were foliar-sprayed with 60 mM pure proline, mixed in 0.1% (v/v) Tween-20 and LP leaf extract containing 60 mM proline. For pure application, proline (MW = 115.13;

Sigma-Aldrich, Japan) was used. For the extraction of LP leaf extract, fresh green LP leaves were harvested from 2-year-old plants. These plants were grown in 14-L earthen pots filled with a specialized medium of sand, silt, and clay in the ratio of 1:1:1. These pots were kept in a growth chamber with day temperature of 23–25 °C and night temperature of 18–20 °C, while light intensity was 675 µmol m<sup>-2</sup> s<sup>-1</sup>. The leaves were chopped into small pieces (≤3 cm). These pieces were dipped in a beaker containing distilled water at the ratio of 1:5 (w/v) for 36 h. After this, the whole material (water + chopped leaf pieces) was blended with an electric blender (Blender-Vivacio, Moulinex, Japan). Blended material was then transferred again to a beaker and kept on shaker (KS 4000i, IKA, Germany) for 5 h at 300 rpm. After shaking, material in the beaker was filtered with a double layer of cheese cloth and the filtrate was filtered through Whatman No. 1 filter paper. This final filtrate was used for experimentation as foliar spray. Proline in filtrate was estimated by the procedure of Bates et al. (1973). The proline concentration measured was 60 mmol kg<sup>-1</sup> plant material. The extract was prepared 1 day before its application as foliar spray. The concentration of LP leaf extract at the ratio of 1:5 (w/v) was selected from a preliminary study (data not shown). In this study, LP leaf extract was taken in distilled water at different ratios, i.e. 1:5, 1:6, 1:7, 1:8, 1:9, and 1:10 (w/v). These extracts were foliar-applied on pea seedlings grown under NaCl (100 mM) and/or NiCl<sub>2</sub> (100 µM) stress in small plastic pots. The best extraction ratio (1:5 v/w) was selected on the basis of maximum improvement in seedling root length, shoot length, and fresh and dry biomass under both stress (Ni and NaCl) conditions. For this reason, the extract (1:5 w/v) was used as a natural source of proline for foliar application. The concentration of proline in this extract was 60 mmol kg<sup>-1</sup> plant material. This concentration of proline in leaf extract was determined by the protocol of Bates et al. (1973). Hence, the concentration of pure proline was also kept the same (60 mM). In another preliminary experiment in small plastic pots (data not shown) regarding the concentrations of NaCl and NiCl<sub>2</sub>, it was observed that concentrations higher than 100 mM and 100 µM, respectively, proved lethal to plant growth. Therefore, a dose below mortal concentration (i.e. 100 mM and 100 µM) was selected for the experimentation. After 2 weeks of foliar spray, samples were collected to record the data for following attributes.

### 2.1. Plant growth attributes

The plants were gently uprooted and washed with distilled water to remove sand particles. The plants were then blotted with filter paper to remove any water present on the leaves and shoots and then placed on a digital balance for the measurement of fresh weights by using a meter scale. The plants were then oven-dried (Memmert-110, Germany) at

72 °C for 3 days and the dry weight was recorded. The leaf area was calculated using a leaf area meter (LI-3100; LICOR, Inc., USA) and 9 leaves per plant were used. These 9 leaves were selected from the 3 upper, 3 middle, and 3 lower portions of the plants used for measuring leaf area.

## 2.2. Chlorophyll contents, gas exchange, and relative water content

Total chlorophyll content was estimated using the method of Arnon (1949). For the measurement of physiological attributes, such as intercellular CO<sub>2</sub> concentration (C<sub>i</sub>), net photosynthesis rate (P<sub>n</sub>), and stomatal conductance (g<sub>s</sub>), 3 young, fully developed, and healthy leaves per plant were selected and placed individually in the chamber of a portable infrared gas analyzer (Analytical Development Company, UK). All recordings of C<sub>i</sub>, P<sub>n</sub>, and g<sub>s</sub> were taken at times between 1000 and 1200 hours with the following conditions: leaf chamber volume gas flow rate, 284 mL min<sup>-1</sup>, molar air flow per unit leaf area, 373.9 mmol m<sup>-2</sup> s<sup>-1</sup>; atmospheric pressure, 96.2 kPa; water vapor pressure in the chamber, 6.4–8.2 mbar; photosynthetic active radiation at the leaf surface, maximum 1589 μmol m<sup>-2</sup> s<sup>-1</sup>; leaf temperature, 24.1 °C; ambient temperature, 22.1 °C; and ambient CO<sub>2</sub> concentration, 322 μmol mol<sup>-1</sup>.

The mature leaves were detached from plants and were tagged, washed under tap water for at least 5 min, blotted with tissue paper, weighed, kept in tap water for 24 h, and weighed again to calculate turgid weight (TW). The leaves were then oven-dried at 72 °C for 48 h and their dry weight (DW) was measured on a digital electrical balance (Bosch AE-160, Germany). Relative water content (RWC) was calculated by the method of Barrs and Weatherley (1962).

$$RWC = \frac{FW-DW}{TW-DW} \times 100$$

## 2.3. Osmolytes, lipid peroxidation, membrane stability index, electrolyte leakage, and free radicals of oxygen

The free proline contents of leaves were estimated by the method of Bates et al. (1973). The glycine betaine content was determined by the method of Grieve and Grattan (1983). Total free amino acids were estimated by the protocol of Hamilton and Van Slyke (1943). The method of Riaz et al. (1985) was adopted to determine the total soluble sugars. Total phenolics were determined by protocol of Julkunen-Tiitto (1985). Total tocopherol contents were recorded according to the procedure of Lee et al. (2007). Lipid peroxidation was estimated by measuring the concentration of malondialdehyde (MDA) and thiobarbituric acid (TBA) as per the method of Heath and Packer (1968). Equal volumes of leaf extract and 0.5 % (w/v) TBA solution with 20% (w/v) trichloroacetic acid (TCA) were mixed. This mixture of fresh leaves, TBA, and TCA was kept at 95 °C for 30 min and then rapidly cooled in a cooling bath filled with ice. The mixture was

centrifuged at 3000 × g for 10 min. After centrifuging, the absorbance of the supernatant at wavelengths of 532 and 600 nm was measured. The MDA concentration was measured from its molar extinction coefficient (155 mM<sup>-1</sup> cm<sup>-1</sup>) and the readings were recorded in MDA g<sup>-1</sup> DM.

$$MDA \text{ level (nmol)} = \Delta (A_{532 \text{ nm}} - A_{600 \text{ nm}}) / 1.56 \times 105$$

For the estimation of membrane stability index (MSI), plant material (250 mg of leaf per tube) was taken in test tubes filled with 10 cm<sup>3</sup> of double-distilled water. One set of these test tubes was heated at 40 °C for 30 min in a water bath, and the electrical conductivity of the solution was recorded on a conductivity bridge (C1) (YSI-31, YSI Inc., USA). The second set of test tubes was heated at 100 °C in a boiling water bath for 10 min, and conductivity was measured on a conductivity bridge (C2). Finally, MSI was calculated by the following formula:

$$MSI = [1 - (C1/C2)] \times 100.$$

The total inorganic ions leaked from the leaves were estimated by the method of Sullivan and Ross (1979). Leaf disks were put in a test tube with boiling deionized water (10 mL) and electrical conductivity was measured (E<sub>cA</sub>). After that, the material was subjected to temperatures of 45 and 55 °C for 30 min each in a water bath and electrical conductivity (E<sub>cB</sub>) was recorded. The material within the test tubes was again boiled at 100 °C for 10 min and electrical conductivity was noted (E<sub>cC</sub>). The electrolyte leakage was measured by using the following formula:

$$\text{electrolyte leakage (\%)} = \frac{E_{cB} - E_{cA}}{E_{cC}} \times 100.$$

The superoxide and hydrogen peroxide generation rate was calculated by the procedures of Elstner and Heupel (1976) and Patterson et al. (1984), respectively.

## 2.4. Polyamine contents

Polyamines were measured by the protocol of Sharma and Rajam (1995). The leaf material was homogenized in 3.2 mL of 5% (w/v) cold perchloric acid (PCA) and incubated at 4 °C for 60 min, and then 1,6-hexanediamine was mixed with the homogenate as the internal standard. After that, the homogenate was centrifuged at 12,000 × g for 30 min at 4 °C. The supernatant was used to measure free and soluble-conjugated polyamine contents, whereas the pellet was used to estimate insoluble-bound polyamines, by the methods given below. For the measurement of conjugated polyamines, a mixture of 1 mL of PCA and 5 mL of 6 N HCl was subjected to hydrolysis. After this, HCl was removed by heating at 70 °C and the remaining

material was centrifuged as indicated above. This solution contained the acid-soluble polyamine fraction having free polyamines. For measuring insoluble-bound polyamines, the pellet was rinsed 4 times with 5% PCA to eliminate any soluble polyamines and mixed with 5 mL of 6 N HCl. This mixture was hydrolyzed by the same method used for measuring soluble-conjugated polyamines and served as the insoluble-bound polyamine fraction. Polyamines recovered from hydrolyzed supernatant, nonhydrolyzed supernatant, and the pellet were benzoylated with benzoyl chloride. The solution was vortexed and incubated for 30 min at 37 °C. The benzoyl polyamines were extracted by adding 4 mL of saturated NaCl solution followed by the addition of 2 mL of cold diethyl ether. Finally, 1 mL of the ether phase was evaporated to dryness and redissolved in 100 mL of methanol. Polyamines were examined by high-performance liquid chromatography. Polyamine peaks were detected with a UV detector at 254 nm. Soluble-conjugated polyamine contents were recorded by subtracting the free polyamine contents from the acid-soluble polyamine contents.

**3. Results**

NaCl (100 mM) and/or NiCl<sub>2</sub> (100 µM) stress applied through the root medium markedly reduced the shoot and root fresh and dry weights and leaf area in pea plants (Table 1). The mutual effect of both stresses was more drastic compared to the individual ones. However, the foliar

spray with LP leaf extract, in the absence of salinity and/or nickel stress, improved growth (P = 0.05). Of both proline sources, LP leaf extract was highly beneficial in promoting the growth of pea plants under nickel and/or salinity stress and the values were markedly higher than those of the plants grown under stress alone (NaCl or NiCl<sub>2</sub>). The plants subjected to combined stress (NaCl + NiCl<sub>2</sub>) and exogenously treated with a natural source of proline showed better improvement in the above indicated attributes than the plants treated with pure synthetic proline. The plants grown under combined stress (nickel and salinity) and sprayed with LP leaf extract exhibited plant fresh and dry biomass and leaf area values higher than those of the plants exposed to Ni and/or salinity stress with no foliar spray. The stress yielded by NaCl and/or NiCl<sub>2</sub> caused a significant reduction in the total chlorophyll contents. The combined effect of both treatments was more deleterious compared to their individual effects. However, this was improved by the foliar application of both pure proline and naturally enriched LP leaf extract, both under stressed and nonstressed conditions. Of both proline sources, the effect of LP leaf extract was better than that of pure proline when applied under NaCl and/or NiCl<sub>2</sub> and combined stressed conditions.

Photosynthetic attributes (Ci, Pn, and gs) were significantly reduced in the plants exposed to NaCl and/or NiCl<sub>2</sub> (Table 1). In the case of Pn and gs, nickel stress was more drastic than NaCl, and their collective effect

**Table 1.** Effect of proline (60 mM) and *Lolium perenne* leaf extract (containing 60 mM proline) (LPLE) on plant fresh weight (g), plant dry weight (g), leaf area (cm<sup>2</sup>), total chlorophyll (mg g<sup>-1</sup> FW), intercellular CO<sub>2</sub> (Ci) (ppm), stomatal conductance (gs) (mmol m<sup>-2</sup> s<sup>-1</sup>), photosynthetic activity (Pn) (µmol CO<sub>2</sub> m<sup>-2</sup> S<sup>-1</sup>), and relative water content (RWC) (%) contents in pea (*Pisum sativum* L.) subjected to NiCl<sub>2</sub> (100 µM) and/or NaCl (100 mM) stress.

Treatments	Plant fresh weight	Plant dry weight	Leaf area	Total chlorophyll contents	Ci	gs	Pn	RWC
Control	14.46cd	3.65bcd	10.99bc	2.49b	199.01b	63.82bc	40.38bc	74.89b
Proline	16.40b	5.04a	14.22a	2.77a	241.53a	69.09b	45.79ab	82.45a
LPLE	18.50a	5.63a	15.14a	3.05a	253.75a	77.55a	49.28a	85.21a
NaCl	9.65g	2.44fg	6.54f	1.77fg	139.88ef	46.83ef	24.52fg	51.59g
NiCl <sub>2</sub>	9.75g	2.52efg	6.22fg	1.78fg	152.32de	42.98f	23.63g	56.26f
NaCl + NiCl <sub>2</sub>	8.23h	1.82g	5.39g	1.40h	118.30f	33.75g	14.23h	45.88h
NaCl + proline	14.02cd	3.69bcd	10.03cd	2.05def	165.28cd	49.99e	30.06ef	66.59cd
NiCl <sub>2</sub> + proline	13.41de	3.60bcd	9.53d	2.11cde	170.23cd	51.85de	33.19de	65.20d
NaCl + LPLE	15.00bc	4.15b	11.10b	2.29bcd	180.33bc	63.15bc	35.00cde	69.59c
NiCl <sub>2</sub> + LPLE	14.90c	4.01bc	10.93bc	2.39bc	177.98bc	57.72cd	38.64cd	73.71b
NaCl + NiCl <sub>2</sub> + proline	11.07fg	3.05def	7.90e	1.74g	153.54de	43.56f	21.57g	57.54f
NaCl + NiCl <sub>2</sub> + LPLE	12.04ef	3.30cde	8.33e	1.89efg	163.31cd	47.83ef	26.17fg	61.13e
LSD at P = 0.05	1.42	0.78	1.00	0.29	21.86	6.40	5.92	3.35

The values are the means of 5 independent replicates. Means in each column sharing the same letter are nonsignificant at P = 0.05.

was more toxic than their individual effects. Regarding Ci and RWC, salinity stress generated more inhibiting effects than nickel stress. The toxicity produced due to the exposure to nickel and/or salinity stress was mitigated by foliar application of synthetic and natural proline; however, the plants treated with natural proline exhibited more melioration in photosynthetic attributes and RWC in comparison to the stressed plants foliar-sprayed with synthetic proline.

A varied trend of response was noted when superoxide, hydrogen peroxide, lipid peroxidation, MSI, and electrolyte leakage were investigated in proline-treated (synthetic and natural) plants in the presence and absence of nickel and/or salinity stress (Table 2). The collective effects of NiCl<sub>2</sub> and NaCl induced more superoxides, hydrogen peroxides, lipid peroxidation, and electrolyte leakage compared to their individual effects. Similarly, plants subjected simultaneously to both salinity and nickel stress had a greater reduction in MSI than those exposed to NaCl and NiCl<sub>2</sub> individually. The unstressed plants sprayed with pure proline and proline-enriched leaf extract of LP did not exhibit any changes in superoxides, lipid peroxidation, MSI, or electrolyte leakage. On the other hand, foliar-applied proline sources significantly enhanced MSI and decreased superoxides, hydrogen peroxides, lipid peroxidation, and electrolyte leakage under stressed

conditions. LP leaf extract in particular exhibited highly beneficial results by markedly lowering lipid peroxidation and electrolyte leakage while improving the MSI under stressed conditions.

The presence of NaCl and/or NiCl<sub>2</sub> in the rooting medium caused a significant increase in the activities of polyamine biosynthetic enzymes [arginine decarboxylase (ADC), ornithine decarboxylase (ODC), and S-adenosylmethionine decarboxylase (SAMDC)] compared to the control (Table 2). Nickel stress triggered ADC and SAMDC activities more than salinity stress; however, in the case of ODC activities, the individual effects of both stresses were not significantly different from each other. The combined application of both stress factors induced maximum acceleration in the functioning of the aforementioned enzymes in comparison to their single application. However, the follow-up treatment of the stressed plants with synthetic and natural proline further increased the activities of ADC, ODC, and SAMDC. Of the treatments, it was found that foliar-applied natural proline (LP leaf extract) increased the activities of these polyamine biosynthetic enzymes more than the pure proline (synthetic).

Leaf free polyamine contents, i.e. Spm, Spd, and Put, were significantly increased under stressed conditions (Table 3). The plants facing NiCl<sub>2</sub>-stress had more elevation

**Table 2.** Effect of proline (60 mM) and *Lolium perenne* leaf extract (containing 60 mM proline) on superoxide production rate (nmol min<sup>-1</sup> g<sup>-1</sup> DW), hydrogen peroxide content (μmol g<sup>-1</sup> DW), lipid peroxidation (nmol mL<sup>-1</sup> g<sup>-1</sup> FW), membrane stability index (MSI) (%), electrolyte leakage (%), arginine decarboxylase activity (ADC) (μL CO<sub>2</sub> g<sup>-1</sup> DW min<sup>-1</sup>), ornithine decarboxylase activity (ODC) (μL CO<sub>2</sub> g<sup>-1</sup> DW min<sup>-1</sup>), and S-adenosylmethionine activity (SAMDC) (μL CO<sub>2</sub> g<sup>-1</sup> DW min<sup>-1</sup>) in pea (*Pisum sativum* L.) subjected to NiCl<sub>2</sub> (100 μM) and/or NaCl (100 mM) stress.

Treatments	Superoxide production rate	Hydrogen peroxide content	Lipid peroxidation	MSI	Electrolyte leakage	ADC	ODC	SAMDC
Control	6.40f	51.25i	9.44e	57.76a	10.49h	93.97l	111.81h	172.64j
Proline	6.49f	52.06i	9.16e	58.92a	10.38h	102.88k	109.07h	215.45i
LPLE	6.36f	40.91j	9.40e	60.77a	10.45h	110.44j	110.11h	249.50h
NaCl	28.31b	169.89d	16.34b	37.73de	28.67b	144.69i	129.30g	254.13h
NiCl <sub>2</sub>	28.69b	185.11c	16.75b	36.84de	27.61c	153.45h	132.14g	264.84g
NaCl + NiCl <sub>2</sub>	36.08a	246.05a	18.22a	24.04f	33.74a	184.79g	165.66f	309.93f
NaCl + proline	20.11c	102.60f	14.13d	49.04bc	15.71f	200.71e	190.93d	325.39e
NiCl <sub>2</sub> + proline	19.77c	114.30e	13.91d	48.24c	16.31f	196.54f	185.10e	325.09e
NaCl + LPLE	10.11e	74.12g	9.75e	54.92ab	11.03h	231.44d	270.03c	363.05d
NiCl <sub>2</sub> + LPLE	11.20d	64.64h	9.94e	56.72a	12.72g	251.72c	271.65c	370.54c
NaCl + NiCl <sub>2</sub> + proline	29.30b	196.30b	15.38c	32.38e	21.63d	270.29b	306.97b	398.20b
NaCl + NiCl <sub>2</sub> + LPLE	29.12b	172.66d	14.70cd	39.53d	18.05e	300.52a	332.88a	418.70a
LSD at P = 0.05	0.99	4.51	0.88	5.90	0.90	3.90	3.16	5.86

The values are the means of 5 independent replicates. Means in each column sharing the same letter are nonsignificant at P = 0.05.

**Table 3.** Effect of proline (60 mM) and *Lolium perenne* leaf extract (containing 60 mM proline) on free, soluble-conjugated, and insoluble-bound polyamines contents (spermine: Spm, spermidine: Spd, putrescine: Put) ( $\mu\text{mol g}^{-1}$  DW) in the leaves of pea (*Pisum sativum* L.) subjected to  $\text{NiCl}_2$  (100  $\mu\text{M}$ ) and/or NaCl (100 mM) stress.

Treatments	Free			Soluble-conjugated			Insoluble-bound		
	Spm	Spd	Put	Spm	Spd	Put	Spm	Spd	Put
Control	0.42i	3.48i	4.27gh	0.044h	0.62i	1.00i	0.036i	0.55i	0.46i
Proline	0.60h	4.32h	4.21h	0.090g	0.90h	0.99i	0.044hi	0.70h	0.49i
LPLE	0.77g	4.98g	4.24h	0.112fg	1.01g	1.01i	0.066gh	0.83g	0.51i
NaCl	0.83g	5.52f	4.53fg	0.130fg	1.07f	1.71h	0.088fg	0.99f	0.78g
$\text{NiCl}_2$	1.02f	5.03g	4.55f	0.132f	1.03fg	1.83g	0.098ef	1.01f	0.72h
NaCl + $\text{NiCl}_2$	1.08e	6.35e	4.88e	0.212e	1.19e	2.10f	0.112de	1.19e	1.06f
NaCl + proline	1.17d	7.07d	5.33d	0.320d	1.32d	2.18e	0.128cd	1.21e	1.24e
$\text{NiCl}_2$ + proline	1.18d	6.93d	5.37d	0.330d	1.29d	2.14ef	0.136c	1.23e	1.30d
NaCl + LPLE	1.25c	7.54c	6.06c	0.400c	1.48c	2.69d	0.150bc	1.41c	1.67c
$\text{NiCl}_2$ + LPLE	1.29c	7.63bc	6.15c	0.492b	1.51c	2.77c	0.160b	1.36d	1.71c
NaCl + $\text{NiCl}_2$ + proline	1.37b	7.98ab	6.56b	0.520b	1.62b	3.01b	0.170ab	1.72b	2.06b
NaCl + $\text{NiCl}_2$ + LPLE	1.45a	8.27a	7.08a	0.572a	1.70a	3.19a	0.184a	2.01a	2.38a
LSD at P = 0.05	0.06	0.40	0.27	0.040	0.05	0.067	0.022	0.05	0.054

The values are the means of 5 independent replicates. Means in each column sharing the same letter are nonsignificant at P = 0.05.

in free Spm and Put contents, while Spd content was high in plants under NaCl stress. However, the combined effect of both stresses (nickel and salinity) caused a greater increase in the ratios of leaf free polyamines than they did individually. Both foliar-applied proline sources further elevated the free polyamine contents in the leaves of plants subjected to salinity and/or nickel stress. LP leaf extract showed better performance under nickel and/or salinity stress in terms of high free polyamines in stressed plants.

Soluble-conjugated polyamine contents (Spm, Spd, and Put) in the leaves of plants grown under NaCl and/or  $\text{NiCl}_2$  regimes were considerably higher than those of plants grown under nonstressed conditions (Table 3). The nickel stress increased soluble-conjugated Spm and Put content, but salinity stress improved soluble-conjugated Spd content. Nevertheless, the mutual effect of both stress factors (NaCl and  $\text{NiCl}_2$ ) induced the maximum accumulation of soluble-conjugated polyamines in leaves as compared to their individual effects. Both proline sources varied in leaf soluble-conjugated polyamine contents under stressed conditions, but natural proline caused a greater accumulation of soluble-conjugated polyamines than that of pure proline (synthetic). However, foliar-applied proline from both sources (natural and synthetic) did not show any significant effect in the case of soluble-conjugated Put under nonstressed conditions.

The plants exposed to nickel and/or salinity stress exhibited higher concentrations of leaf insoluble-bound

polyamines, i.e. Spm, Spd, and Put, in comparison to the control (stress-free conditions) (Table 3). Regarding the individual effect of both stress factors,  $\text{NiCl}_2$  resulted in the maximum increase in insoluble-bound Spm, while NaCl induced high accumulations of insoluble-bound Put. In spite of this, nickel and salinity stresses were equal in their effects for insoluble-bound Spd. However, it was observed that plants submitted to simultaneous stress of NaCl and  $\text{NiCl}_2$  had higher insoluble-bound polyamines than those treated with NaCl or  $\text{NiCl}_2$  separately. The subsequent treatment of the stressed plants with natural and pure proline enhanced leaf insoluble-bound polyamines. The plants treated simultaneously with both NaCl and  $\text{NiCl}_2$  and sprayed with proline (LP leaf extract or synthetic) were higher in leaf insoluble-bound polyamines. Of both proline sources, the stressed plants foliar-treated with LP leaf extract had better improved insoluble-bound polyamines in leaves.

The levels of free proline, glycine betaine, total free amino acids, total soluble sugars, total phenolic contents, and tocopherol content were elevated in response to NaCl and/or  $\text{NiCl}_2$  stress in leaves compared to the nonstressed plants (Table 4). The plants facing the mutual effects of nickel and salinity presented the highest levels of the above-indicated osmolytes in their leaves, higher than that of the individual effects of NaCl and/or  $\text{NiCl}_2$ . Both proline sources (synthetic and natural) further augmented the organic osmolytes in the plants grown under  $\text{NiCl}_2$

**Table 4.** Effect of proline (60 mM) and *Lolium perenne* leaf extract (containing 60 mM proline) on proline contents (mg g<sup>-1</sup> DW), glycine betaine contents (mg g<sup>-1</sup> DW), total free amino acids (mg g<sup>-1</sup> DW), total soluble sugars (mg g<sup>-1</sup> DW), total phenolic contents (mg g<sup>-1</sup> DW), and tocopherol contents (µg g<sup>-1</sup> DW) in the leaves of pea (*Pisum sativum* L.) subjected to NiCl<sub>2</sub> (100 µM) and/or NaCl (100 mM) stress

Treatments	Proline	Glycine betaine	Total free amino acids	Total soluble sugars	Total phenolics	Total tocopherols
Control	1.13i	1.49g	1.44j	4.34k	8.70h	2.56i
Proline	1.77h	1.68g	1.66i	5.28j	10.13g	2.99gh
LPLE	2.08gh	1.62g	1.89h	6.13i	11.05f	2.78hi
NaCl	2.41fg	3.34f	2.06g	7.63g	11.01f	3.30fg
NiCl <sub>2</sub>	2.58ef	3.44ef	2.13f	7.12h	11.10f	3.13gh
NaCl + NiCl <sub>2</sub>	3.06bc	4.35bc	2.39e	8.36f	12.15e	3.63ef
NaCl + proline	2.77cde	3.82def	2.65d	9.02e	13.47cd	3.74de
NiCl <sub>2</sub> + proline	2.70def	3.86c-f	2.68d	9.33e	13.09d	3.99cde
NaCl + LPLE	3.01bcd	4.03cd	3.07c	10.37d	13.93bc	4.11bcd
NiCl <sub>2</sub> + LPLE	3.06bc	3.96cde	3.11c	10.72c	13.88bc	4.23bc
NaCl + NiCl <sub>2</sub> + proline	3.28ab	4.67ab	3.29b	11.71b	14.33b	4.47ab
NaCl + NiCl <sub>2</sub> + LPLE	3.40a	5.04a	3.49a	12.09a	15.26a	4.71a
LSD at P = 0.05	0.34	0.53	0.07	0.35	0.57	0.41

The values are the means of 5 independent replicates. Means in each column sharing the same letter are nonsignificant at P = 0.05.

and/or NaCl stress. However, the maximum concentration of these osmolytes was recorded in the plants grown under both nickel and salinity (combined) stress and subsequently treated with LP leaf extract.

#### 4. Discussion

In this study, a considerable reduction in plant fresh/dry biomass and leaf area of pea plants exposed to nickel and/or salinity stress was observed. These results are similar to what has already been documented for pea (Ormrod, 1977; Maidanyuk et al., 1980; Shahid et al., 2012, 2013). Besides many other factors, inhibition in cell growth and cell death are also reasons for suppression in growth under stress conditions. The rate of cell death was not investigated in the present study, but reduction in growth may be associated with this fact. However, both foliar-applied proline sources significantly elevated pea growth under stress generated by nickel and/or salinity. The promotion of growth in response to exogenous application of proline (both synthetic and natural) could have been due to the decline in the rate of cell death under stressed conditions. Islam et al. (2009) and Banu et al. (2009) also found that exogenous proline inhibited the rate of cell death under Cd and salinity stress. The findings of the present study regarding the role of proline are also in accordance with some previous investigations in which it was found that proline overcame growth suppression caused by various stress factors in different plant genotypes (Hoque et al.,

2007; Islam et al., 2009; Ozden et al., 2009; Soshinkova et al., 2013). On the other hand, the literature shows that polyamines are actively involved in promoting cell division and reducing cell death (Galston and Kaur-Sawhney, 1987; Thomas and Thomas, 2001). In the current study, proline-induced (natural or synthetic) growth under NaCl and/or NiCl<sub>2</sub> could have been linked to regulation in cell division, under the effect of increased endogenous polyamines in leaves.

A marked decline in the photosynthetic rate of pea plants was observed under NaCl and/or NiCl<sub>2</sub> stress in the current research; exogenous application of pure proline and LP leaf extract alleviated the stress-induced deleterious effects on the photosynthetic rate of pea. It is also interesting that, in the present investigation, plant fresh and dry biomass was positively associated with photosynthetic capacity, indicating that foliar-applied proline elevated growth by increasing photosynthesis. The rate of stomatal conductance is highly linked with the rate of photosynthesis, which is a marked indication of enhanced photosynthetic activity by the regulating of stomatal conductance. The findings of Ben Ahmed et al. (2010) and Hayat et al. (2013) also support the results of the present study. It was reported that polyamines are also involved in the functioning of stomata by regulating Ca<sup>2+</sup> homeostasis in the cytoplasm of guard cells (Liu et al., 2000; Yamaguchi et al., 2006). Therefore, increased stomatal conductance and photosynthetic activity in



stressed plants treated with proline (LP leaf extract or pure) could be attributed to enhanced polyamine contents in leaves. Similarly, Hamdani et al. (2011) also reported that polyamines are involved in strengthening the photosynthetic apparatus under stressed conditions.

The results of the present study also show that nickel and/or salinity stress induced a marked decline in total chlorophyll content. The reduction in chlorophyll content may be due to enhanced activity of chlorophyllase (a chlorophyll-degrading enzyme) in a stressed environment (Ozturk et al., 2002; Khan, 2003). Nickel destroys chlorophyll content by replacing the Mg ions of green pigments (Kupper et al., 1998), suppressing the activities of enzymes involved in chlorophyll formation (Shalygo et al., 1999) and accelerating the activities of chlorophyllase (Abdel-Basset et al., 1995). All these effects culminate into a reduction in total chlorophyll content. On the other hand, the degradation of biological membranes in chloroplast due to the specific ion's toxicity ( $\text{Na}^+$  and  $\text{Cl}^-$ ) cannot be ignored, because the more toxic ions there are in the leaf tissues, the greater the decrease in total chlorophyll content is. In the current investigation, total chlorophyll content exhibited a positive linkage with foliar-applied pure proline or LP leaf extracts, which indicates that changes in photosynthesis due to the spray of pure proline or LP leaf extract can be attributed to improvement in chlorophyll pigments. On the other hand, it was found that polyamines, especially Spm, have a significant effect in restoring chlorophyll contents under stressed conditions (Hamdani et al., 2011). Since both proline sources markedly enhanced the leaf polyamine contents, including Spm, in plants exposed to  $\text{NiCl}_2$  and/or  $\text{NaCl}$  stress, the improvement in total chlorophyll contents in the current study may be pertinent.

The pea plants showed a significant elevation in endogenous organic osmolytes, i.e. free proline, glycine betaine, total free amino acids, total soluble sugars, and total phenol and tocopherol contents in leaves, upon exposure to  $\text{NaCl}$  and/or  $\text{NiCl}_2$  stress. This increase in endogenous osmolytes resulted in high osmotic adjustment potential. Foliar application of proline or LP leaf extract enhanced the concentration of endogenous compatible solutes and consequently caused an augmentation in osmotic adjustment capacity. The improved osmotic adjustment potential in terms of increased organic osmolytes resulted in increased MSI, RWC, stomatal conductance, and photosynthetic activity. Overall, increased nickel and/or salinity tolerance capacity in tested pea plants in response to foliar-applied proline or LP leaf extract is attributed to high osmotic adjustment potential, due to an increase in endogenous osmolytes.

Generation of reactive oxygen species (ROS) in excessive amounts is a common consequence of various stresses,

including salinity and/or nickel. To regulate the metabolic processes under stress conditions, there should be a balance between the rates of formation and degradation of ROS; otherwise, oxidative stress may occur (Apel and Hirt, 2004). To overcome oxidative damage under stressed conditions, plants develop an antioxidant defense system consisting of various antioxidant enzymes (Apel and Hirt, 2004) and nonenzymatic antioxidants e.g., proline, tocopherols, glycine betaine, total soluble sugars, total free amino acids, phenolic compounds, ascorbic acid, and carotenoids (Mittler et al., 2004; Nazar et al., 2008). This antioxidant system maintains ROS at a less toxic level by converting them into water and oxygen. Therefore, it was anticipated that pea plants exposed to  $\text{NaCl}$  and/or  $\text{NiCl}_2$  stress could enhance the ratios of nonenzymatic antioxidants such as proline, glycine betaine, total free amino acids, total soluble sugars, total phenolic compounds, and tocopherol contents in their leaves. Low values of free radicals (superoxide and hydrogen peroxide) and lipid peroxidation were noted in the plants exposed to combined stress generated by nickel and salinity and then subjected to applications of proline and LP leaf extract. Of both proline sources, LP leaf extract gave better results than pure proline. The reduction in the production of superoxide and hydrogen peroxide led to an improvement in MSI and a decline in electrolyte leakage in the current study.

Polyamines are highly associated with defensive mechanisms of plants against abiotic stresses (Bitrian et al., 2012). Generally, plant genotypes with high stress-tolerance capacity have the maximum potential to elevate polyamine biosynthesis in response to various abiotic stresses, including salinity and nickel stress. Under stressed conditions, polyamines play a major role in maintaining a balance between ratios of cations and anions (Jantaro et al., 2003; Tonon et al., 2004). The plants submitted to  $\text{NaCl}$  and/or  $\text{NiCl}_2$  stress exhibited a marked increase in leaf polyamine content. Applications of proline (natural and synthetic) improved polyamine metabolism by enhancing free, conjugated-soluble, and insoluble-bound polyamine (Spm, Spd, and Put) contents in the leaves of stressed plants. This increase in leaf polyamine contents could have been due to the increased activities of polyamine biosynthetic-enzymes (ADC, ODC, and SAMDC) in response to the exogenous application of proline (LP leaf extract or pure). The enhanced concentration of leaf polyamine content in plants grown under stressed conditions and foliar-treated with LP leaf extract or pure proline is responsible for reducing the oxidative injuries generated due to  $\text{NiCl}_2$  and/or  $\text{NaCl}$  stress by scavenging ROS, i.e. superoxide and hydrogen peroxide radicals. Polyamines strengthen the defensive mechanisms against stresses within plants by accelerating antioxidant and nonantioxidant (organic osmolytes) activities, thereby suppressing the generation

of free radicals of oxygen that destroy cellular membranes. Therefore, it is suggested that the high polyamine content in the leaves of stressed plants under foliar application of proline might be responsible for improvement in MSI, resulting in efficient photosynthetic activity.

Overall, exogenously applied proline (pure and natural) significantly improved the growth of stressed pea plants; however, LP leaf extract was more efficacious than pure proline. The mechanism of LP leaf extract's effectiveness is not elucidated yet, but it is suggested that the presence of various essential nutrients in LP leaf extract may explain its higher efficiency when compared to pure proline. The growth augmentation in response to foliar-applied proline or LP leaf extract was found to be associated with increased levels of endogenous polyamines and organic osmolytes

in leaves, which led to improvements in photosynthetic capacity, total chlorophyll content, RWC, MSI, and suppression of free radicals of oxygen. As a natural source of proline, LP leaf extract was as beneficial as pure proline in improving growth, selected major physiological processes, and polyamine metabolism under nickel and/or salinity stress. Therefore, it can be used as an alternative inexpensive source of proline for safeguarding plants from the phytotoxic effects of nickel and/or salinity stress.

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