# Effect of Sodium Nitroprusside (SNP) and Salicylic acid (SA) on Morphological,Biochemical and Antioxidant Enzymes Activity on Field Pea(*Pisum sativum* L.) under Cadmium Stress

काशी हिन्दू विश्वविद्यालय

BANARAS HINDU UNIVERSITY

**THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF**

**Master of Science (Agriculture)**
**In**
**Plant Physiology**


**Advisor-cum-Chairman**
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**Submitted by**
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# CERTIFICATE

To,
**The Registrar**, **(Academic)**
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**Through**: The Head, Department of Plant Physiology, Institute of Agricultural Sciences, Banaras Hindu University.

Dear Sir,

I have great pleasure in forwarding the thesis entitled, **"Effect of Sodium Nitroprusside & Salicylic acid on Morphological, Biochemical Parameters and Antioxidant enzymes Activity on field Pea (*Pisum sativum* L.) Under Cadmium stress"** Submitted by **Mr. Saurabh Kumar Singh, ID. No-16PPH07** in partial fulfillment of the requirements for the Degree of **Master of Science (Agriculture) in Plant Physiology,** Institute of Agricultural Sciences, Banaras Hindu University, Varanasi and placing on record that he has completed the requisite residential requirements as contained in the ordinance of the University.

I hereby certify that the candidate has devoted his four continuous semesters and the experimental work was carried out by him under my guidance and supervision. To the best of my knowledge and belief, the data incorporated in this thesis are genuine and original and no part of the thesis has been submitted for any other degree or distinction.

Yours faithfully,

***(Padmanabh Dwivedi)***

**Head of the Department**

Advisor-cum-Chairman

# Ameliorative effect of Salicylic acid& Sodium Nitroprusside on Morphological, Biochemical Parameters and Antioxidant enzymes Activity on field Pea (*Pisum sativum* L.) under Cadmium stress


**By**

*Saurabh Kumar Singh*

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**Date:**
**Place: Varanasi**. **(Mr. Saurabh Kumar Singh)**

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# ABBREVIATIONS

| | | |
|---|---|---|
| CD | : | Critical Difference |
| SEm | : | Standard Error Mean |
| FW | : | Fresh Weight |
| USDA | : | United States Deptt. Of Agriculture |
| MT | : | Metric Tonne |
| % | : | Percent |
| Ha | : | Hectare |
| gm | : | Gram |
| cm | : | Centimeter |
| ppm | : | Part per million |
| ng | : | Nanogram |
| Ni | : | Nickel |
| Mg | : | Milligram |
| mM | : | Millimolar |
| m | : | Meter |
| L | : | Liter |
| T | : | Treatment |
| V | : | Variety |
| *et al.* | : | and others |
| i. e. | : | that is |
| Kg | : | Kilogram |
| NP | : | Nano Particals |

| | | |
|---|---|---|
| O.D. | : | Optical Density |
| SAT | : | Serine acetyltransferase |
| DAS | : | Days after Sowing |
| ROS | : | Reactive Oxygen Species |
| µM | : | Micromolar |
| ABA | : | Abscissic acid |
| SOD | : | Super oxide Dismutase |
| A | : | Absorbance |
| APX | : | Ascorbate Peroxidase |
| CAT | : | Catalase |
| MDA | : | Malondialdehyde |

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*Chapter I*

# INTRODUCTION

The **field pea** *Pisum sativum*(2n=14) is a type of pea, offen called *P. sativum* sub sp. *arvense* (L.) Also identified as dun (grey-brown) pea, Kapucijner pea, or Austrian winter pea, field peas are one of the old most adopted domesticated crops, cultivated for at least 7,000 years. They are now grown in many countries for both human consumption and stock feed. There are several cultivars and colours including blue, dun (brown), palmet and white. The field pea is differed with the Cowpea (*Vigna unguiculata*) which is normally called the "field pea" in warmer climates. One of the relatives of field pea is garden pea, whose green pods are used as utilised green vegetables. It is a climbing annual legume with weak, vine, and partially succulent stems. Vines often are four to five feet (1.2 to 1.5 m) long, but when grown alone, field pea's weak stems prevent it from growing more than 45 to 60 cm tall. Leaves have two leaflets and a tendril. Flowers are white, pink and purple in colour. Pods having seeds that are large (four thousand seeds/lb), closely round, white, gray, green, or brown. The root system is relatively less dipper and small, but well nodulated.

Generally field pea is green coloured pea in some reasons it is golden yellow or normally purple pod-coloured, widely grown as a winter season vegetable crop. The seeds may be sown as soon as the soil temperature about is 10 °C (50 °F), with the plants growing best at temperatures of 13 to 18 °C. Summer heat of warmer temperate and lowland tropical climates is not suitable for well grown, but do grow well in cold weather climatic condition, high altitude and tropical areas. Many cultivars reach maturity about 60 days after planting. Peas have both low-growing and coiling pattern cultivars. The coiling cultivars grow thin tendrils from leaves that coil around any available support and can climb to be 1–2 m high

In past times, peas were grown mostly for their dry seeds. From plants growing wild in the Mediterranean zone, continued selection since the Neolithic dawn of agriculture improved their yield. In the early 3rd century BC Theophrastus mentions peas among pulses that are tender that's why they shown in late winter.

In North America pea milk is produced and sold as an alternative to cow milk for a variety of reasons

Peas are rich in carbohydrate, but high in fiber, protein, vitamin A, vitamin B6, vitamin C, vitamin K, phosphorus, magnesium, copper, iron, zinc and lutein.] Dry weight is about one-quarter protein and one-quarter sugar. Pea seed peptide fractions have less ability to scavenge free radicals than glutathione, but higher ability to chelate metals and inhibit linoleic acid oxidation

Plant responses to abiotic stresses are infrequent and complex. The intensity and duration of stress causes the complexity of the response, which may be reversible or irreversible (Cramer et al., 2011; Tattersall *et al*., 2007). Plants cope with these adverse conditions by modifying their physiological, biochemical and molecular processes. The extremities of temperature (i.e. high as well as low) and water limiting conditions are jointly caused dehydration and osmotic stress. Cold stress includes chilling temperatures (< 20 ˚C) or freezing temperatures (< 0 ˚C), which negatively affects the productivity of plants (Chinnusamy *et al*., 2007). Besides affecting growth and metabolic rates, cold stress also limits water uptake by the plant, which causes severe dehydration condition (Chinnusamy and Zhu 2002). When high temperature and water deficiency occuring at the same time, also leads to decreases in growth mainly due to effects on metabolic activity of the plant brought about due to decreases in the water potential in cells. The physiological effects of plants in response to cold or drought stress include limiting water loss by stomatal closure, root proliferation as a result to increase their water absorption capacity, biosynthesis of solutes, which regulates in the osmotic adjustment of the plant by osmolytes and activating antioxidant metabolism to protect oxidative stress arising from metabolic defects (Bhargava and Sawant 2013).

We can see that in these days due to industrialisation heavy metal pollution is drastically increases, food items also contains heavy metal which is main causes of soil pollution as a result , harmful to the health of humankind (McLaughlin *et al*., 2009). Scientists and engineers have discovered new efficacious technologies, in which by the help of microorganisms, biomass and some plants are being used for removing polluted zones (Ebbs, Kochian, 1997; Wasay *et al*., 1998). Among heavy metals cadmium is such a poisonous and harmful elements that are injurious for live organs. From biological point of view also not important for plants, animals and human and causes leaf rolls, chlorosis and decreases of upper and lower plant part growth (Smeets *et al*., 2005; Mishra *et al*., 2006). Researches show that cadmium limits the process of germination and growth and various developmental process of seedling (Rascio *et al.*, 1993). Cd treatment with plant parts decreases the root and shoot-growth up to 27% and this inhibition had positive correlation with the reduction of root cells viability (Siroka *et al*., 2004). One of the biochemical alterations occurring in plants subjected to various biological stress conditions such as cadmium as well as other stress is the production of reactive oxygen species that leads to oxidative stress (Cho, and Park, 2000). The ROS has a definite role in lipid peroxidation, membrane are highly damage and consequently lead to plant senescence (Zhang *et al.*, 2003). Furthermore, photosynthesis is very much responsive to Cd because it directly and adversely affects chlorophyll biosynthesis (Gadallah, 1995) and the reasonable development of chloroplast ultra-structure (Stoyanova and Tchakalova, 1997).

Cadmium chloride is white in colour and in crystal form of cadmium and chlorine, with the formula $CdCl_2$. It is a hygroscopic solid that is why its shows highly solubility towards water and low solubility in alcohol. Although it is considered to be ionic, it has specific covalent character in case of bonding . The crystal structure of cadmium chloride, composed of two-dimensional layers of ions, is a reference for describing other crystal structures Cadmium chloride shape is crystals with rhombohedral symmetry. Cadmium iodide, $CdCI_2$, has a very much similar crystal structure to $CdCl_2$. The layers in the two structures are same, but in $CdCl_2$ the chloride ions are organised in a CCP lattice, whereas in $CdCl_2$ the iodide ions are arranged in an HCP lattice.

In running years, Nitric oxide (NO), a biologically active gas, effective in nanomolar concentration (1.0 nmol $L^{-1}$), has been shown to be everywhere in plants and to regulate various physiological and developmental processes. Nitric oxide is involved in germination and initiation of lateral roots (Creus *et al.*, 2005; Sarath *et al.*, 2006; Tewari *et al.*, 2008*a*), delays senescence (Neill *et al.*, 2003), ease Cu toxicity (Tewari *et al.*, 2008*b*), modulates the influx of extracellular $Ca^{2+}$ and action filament organization during cell wall construction in *Pinus bungeana* pollen tubes (Wang *et al.*, 2009), and up-regulates formation of secondary metabolites in the adventitious roots of *Panax ginseng* and *Echinacea purpurea* (Tewari *et al.*, 2007; Wu *et al.*, 2007). External application of NO also down-regulates xanthine oxidase-mediated generation of $O_2^{\bullet-}$ in *Phalaenopsis* flowers (Tewari *et al.*, 2009). NO can mold complexes with transition metal ions in aqueous media (Stamler *et al.*, 1992). Metal–nitrosyl complexes mold under neutral physiological conditions and may act as links between the different redox states of NO (Stamler *et al.*, 1992).

When NO in the form of SNP (sodium nitro prusside) is provided externally it prevent interveinal chlorosis (a typical Fe-deficiency symptom) even in plants supplied with very low (10 µM) Fe (Graziano *et al.*, 2002; Graziano and Lamatinna 2005; Graziano and Lamatinna 2007; Sun *et al.*, 2007).

Salicylic acid (SA) is one of the important what compiled having significant effects on various biological aspects in plants (Raskin, 1995). Researches showed the effect of salicylic acid on growth in Vicia faba (Manthe et al., 1992) and increase in flower size of Campanula (Serek, 1992). When supply of SA externally to different crops has been showed to elicit yield and yield components. A significant increase in production as number of pods and yield has been found in *Vigna radiata* (Sing & Kaur, 1980) and in *Brassica juncea* (Fariduddin et al., 2003). According to Senaratna et al., (2003), SA has a important role in tolerance to heat, drought and chilling stress in bean and tomato plants. Such a multiple functioning of stress tolerance mechanisms have been reported in the literature and contribution that SA has causes great effort in stress tolerance and identified as stress tolerance hormone. SA is one of the endogenous important PGR which regulators, naturally occurring signalling molecule and with major role in growth and development of plants. Salicylic acid is important

compound required in many physiological processes such as photosynthesis, nutrient uptake and transport, flowering and inhibition of fruit ripening.

We are now introduced with the field pea, abiotic stress their effect on different morpho physiological process of plant, heavy metal stresses, their effects and two ameliorates sodium nitroprusside and salicylic acid which counter the harmful effects on plants due to heavy metal stress. The main moto of the research works is to see the interactive result of salicylic acid with $CdCI_2$ stress, sodium nitroprusside with $CdCI_2$ stress, salicylic acid +sodium nitroprusside + $CdCI_2$ stress. What are their effects on different enzymatic activity on the plants, How the growth is affected what happened in the metabolic processes , we have seen hear that how different components of plants such as chlorophyll a ,chlorophyll b ,caratenoids and protein content also estimating the SOD, MDA , APX , $H_2O_2$,MSI and affected due to stress.

The availability of Cd is increased by the application of chloride and reduced by application of silicon even if Cd availability is decreased by adding amendments. Cd in soil causes many disturbances in mineral nutrition and carbohydrate metabolism; reduce biomass, inhibition of chlorophyll synthesis, deleterious effect in photosynthetic processes. Tolerant varieties at the seedling, vegetative and reproductive growth stages sensitive to elevated concentrations of cadmium led to accumulation of cadmium in the shoot and roots

The following objective of this research work:

- Physiological effect of Cd stress on morphological and biochemical parameter in pea plants.
- What are individual effects of these two ameliorates salicylic acid and sodium nitroprusside (NO) on heavy metal stress.

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*Chapter II*

# REVIEW OF LITERATURE

**2.1. Cadmium**.

Generally the source of heavy metal is through soil which is naturally occurring. High concentrations are confined primarily to certain minerals usually present in forms which are not easily available but may be sensitive to higher concentrations (Dmitriev, 1949). The release of heavy metals in biologically available forms, as a result of human activities, may damage both, natural and man-made ecosystems. The chemical form of heavy metals in soil solution is greatly dependent on the metal element concerned, pH and presence of other ions (Pinto *et al*., 2005). Poisonous actions of ions are essentially exerted on the enzymes. Inhibition of enzymes may be due to the making of catalytically active groups or protein denaturation. Prolonged exposure of soils to heavy metals may result in marked decrease in soil enzyme activity (Tyler *et al*., 1989). Cadmium is non-essential heavy metal pollutant of the environment resulting from various agricultural, mining and industrial activities and also from exhaust gases of increases automobiles (Foy *et al.*, 1978). It has been considered as one of the extremely pollutant due to its high toxicity and greater solubility in water which determines wide distribution in aquatic ecosystems (Lockwood, 1976).

The harmful effects of cadmium on biological systems were reported by various authors (Mukherjee *et al.*, 1984, Sharma *et al.*, 1985). Cadmium is found naturally into the ecosystem and other relative factor; it is present in soil and sediments at concentrations which are generally 1 mg $Kg^{-1}$ (Peterson and Alloway, 1979).

Cadmium has been formed to many plant species and plant part, although it is not usually considered to be a micronutrient. However, it has been reported to increase the sugar content and yield of grapes when applied in a foliar spray (Dobrolyubskii, 1957; Dobrolyubskii and Slavvo, 1958) and to increase the yield of

red clover when applied in combination with boron (Dmitriev, 1939). Bertrand and de Wolf (1959) stated that in the fungus *Aspergillus niger*, cadmium may substitute for zinc in the synthesis of tryptophan, an amino acid necessary for the elaboration of indole acetic acid hormone.

## 2.2. Cadmium Toxicity in Higher Plants

Cadmium is recognized as an extremely significant hazardous due to its high toxicity and large solubility in water (Pinto *et al*., 2004). Important sources of cadmium input to the marine environment include atmospheric deposition, domestic waste water and industrial discharges (Benavides *et al*., 2005). Wagner (1993) examined that non-polluted soil solutions contain cadmium concentrations range from 0.05 to 0.36 mM. Soil solutions which have a cadmium concentration varying from 0.32 to about 1 mM can be regarded as polluted to a moderate level (Benavides *et al*., 2005).

In many developing countries it is common to grow vegetables along banks of rivers passing through urban centre's. Waters of such rivers have often been reported to be polluted by heavy metals (Mashauri and Mayo, 1990; Kashem and Singh, 1999; Othman, 2001).

The reduction of biomass by Cd toxicity could be the direct consequence of the inhibition of chlorophyll synthesis (Padmaja *et al*., 1990) and photosynthesis (Bazzaz *et al*., 1975. Baszynski *et al*., 1980). Some studies reported a marked in lowering the photosynthetic rate for different plant species under exposure to Cd stress (Sawhney *et al*., 1990. Sheoran *et al*., 1990a. 1990b). when growing the vegetables plants in medium with high level of Cd showed negative effect in photosynthetic processes, such as chlorophyll content and photosynthesis (Baszynski *et al*., 1980. Padmaja *et al*., 1990. Satyakala, 1997), the activities of related enzymes (Ouariti *et al*., 1997 a., 1997b. Ascencio and Cedeno Maldonado, 1979) and photochemical reaction (Li and Miles, 1975. Skorzynska and Baszynski, 1995).

Recent studies conducted in India have pointed long range atmospheric transport and accumulation of trace elements and their possible role in contaminating

terrestrial environment as well as agro-ecosystems (Pandey & Pandey 2009b, 2009c; Sharma *et al*. 2008; Singh & Agrawal 2005).However, there is shortage of studies plainly addressing the heavy metal pollution of fresh water resources, associated to atmospheric deposition. Use of wastewater to irrigate agricultural lands is one of frequent practice in sub-urban and industrial areas in many parts of the world (Sharma *et al*. 2007, Gupta *et al*. 2008). Waste water irrigation leads to accretion of heavy metals in the soil (Singh *et al*. 2004, Mapanda *et al*. 2005, Sharma, Agarwal and Marshall 2007). Sewage waste has been implicated as a potential source of heavy metals such as Copper (Cu), Cadmium (Cd), Zinc (Zn), Lead (Pb), Nickel (Ni) and Iron (Fe) in the edible and non-edible parts of vegetables (Sharma, Agarwal and Marshall 2006).

### 2.3 Effect of cadmium on growth parameters of plants

Cadmium is not an essential part of plant life. In fact they obstruct plants physiology in many ways. The mobility of this metal in soil–plant system allows its easy uptake in excess by plant so that it will directly or indirectly inhibit the physiological processes like respiration, photosynthesis, transpiration, cell elongation, plant-water relationship, mineral nutrition, nitrogen, and carbohydrate metabolism, leading to poor growth and low biomass (Obata and Umebayashi, 1996). The seed germination and early-seedling growth are important stages in the whole process of plant growth and due to being the most sensitive stage in the plants changing of their environment, have been widely used in environmental bio-monitoring (Herna´ndez, L.E., R. Carpena-Ruiz and A. Ga´rate, 1996).

#### 2.3.1 Effect of cadmium on seed germination

The capability of seeds to germinate readily when conditions are suitable for successful growth and the ability to avoid germination at unsuitable times are essential to the survival of a species (Johnson, 2000).Many plants at seed germination and seedling stages are sensitive to environmental factors. Therefore, the change of plant growth at the germination and seedling stage under heavy metal stress is often regarded as an important key to evaluate plant lenience to heavy metals (Peralta-Videa *et al*., 2002). Seed germination is often judged by radicle emergence through

the seed coat. Research results indicated that if the process of germination is divided from the embryo or seedling growth, it is not highly influenced by heavy metals. The process affected was the actual growth of embryo (Koeppe, 1977). According to Linger *et al*. (2005) the germination rate was 70% for control plants compared to 79% and 77% in the cadmium treated plants and they concluded that cadmium concentration up to 72 mg/kg (soil) had no negative effect on germination in *Cannabissativa* L. The higher germination rate on cadmium contaminated soils was also reported by Seregin and Ivanov (2001). Cadmium unfavorably influenced the germination process of cowpea seeds reported by many researches (Vijayaragavan *et al*., 2011). The pessimistic effect of cadmium on germination rate of *Triticum aestivum* L. seedlings has been reported by Munzuroglu and Geckil (2011).

### 2.3.2. Effect of cadmium on Radicle and Plumule length

Cadmium has bad effects on germination attributes. Radicle length not only decreases but show poor growth also. Thickness of radical was also influenced after having treatment with cadmium. Browning of root tips also occur. Shoot growth also decreases, Cd is an inhibiting factor on root and shoot length growth and the effect of Cd on root length growth is more noticeable than shoot length growth (Bahmani *et al*.).Application of Cd reduces the shoot length in bean and alfalfa (Bhardwaj *et al*., 2009; Aydinalp and Marinova, 2009) and also Mihalescu *et al*. (2010) were of the opinion that the reduction in root length and height of the plant was due to Cd accumulation in *Zea mays*.

Shoot and root length decreases with mounting cadmium concentrations (Bolagure *et al*., 1998; Sharma and Sharma, 1996). They showed the reduction of length, fresh weight of stem, branches and leaves. In other study Ozturk *et al.* (2003) observed that the increase in cadmium supply obviously reduced the shoot and root length in wheat seedling. Similar results were obtained in both plants *Cajanus cajan* and *Trigonella foenum-graecum*. Wang and Zhou (2005) observed that cadmium reduced the elongation of wheat seedling and reported that the root growth inhibition was stronger than shoot growth inhibition. In other study Ouzounidou *et al*. (1997) and Vitoria *et al*. (2001) also observed that the root growth was more severely

affected than shoot growth in wheat seedling by cadmium treatment. Dubey (1998) has also noticed reduction in root and shoot growth. He had also found that the root growth was more severely affected than shoot growth.

### 2.3.3. Effect of cadmium on seedling vigor index

Seedling vigor index (SVI) is the probable of seed germination and sprout size against the toxicity and lenience of metals. Cadmium has a unconstructive end product on vigor index because it directly affects the root growth and accordingly affects the vigor index of seed indirectly. A strong unconstructive effect of Cd on root length and the fresh and dry biomass of *M. truncatula* (Aloui *et al*., 2009; Xu *et al*., 2010), *Medicago sativa* (Peralta *et al*., 2001; Ortega-Villasante *et al*., 2005; Dražic *et al*., 2006) and other plant species (Poschenrieder *et al*., 1989; Roosens *et al*., 2003; Metwally *et al*., 2005;Wójcik *et al*., 2005; Nada *et al*., 2007; Groppa *et al*., 2008; Finger-Teixeira *et al*., 2010).

Several authors reported that the checking of root growth caused by heavy metals may be due to metal interference with cell division, as well as enticement of chromosomal aberrations and abnormal mitosis (Radha *et al*., 2010; Liu *et al*., 2003), which can affect seedling augmentation and explain the inhibition of seedling growth and decrease in seedling vigor index. The incident of heavy metals in soil can role as stresses which causes physiological and biochemical constraints thereby lessening plant vigor and inhibiting plant spreading out (Wu and Lin, 1990).

### 2.3.4. Effect of cadmium on Percent phyto-toxicity

The toxic property of heavy metal on the biological functioning has been reported by various authors. The trace metals exerting toxic effects on plants have been studied for over a century by now but there remains uncertainty within the literature with regards to their concentrations as micronutrients and as gears inducing phytotoxic possessions (Jensen, 1907). For example, Taylor and Foy found 30 μM Cu enough for reducing growth of wheat (*Triticum aestivum* L.) by 50%, whereas, Wheeler *et al*. reported only 0.5 uM Cu required for a 50% growth reduction in the same species. Many plant species readily take up $Cd^{2+}$ ions via roots and it is

transported to the leaves in the same way as the necessary micronutrient metal ions, but $Cd^{2+}$ are not essential for plant growth (Patra *et al*., 2004). According to Isak Rajjak Shaikh *et al.* (2013), phytotoxicity of shoot was decreased at lower concentration (2 mg/L) and increased at higher concentration (10 mg /L) and Phytotoxicity of root was decreased at lower concentration (2 mg/L) and increased at higher concentration (10 mg /L). The percentage phytotoxicity of shoot and root were not greatly affected at lower concentrations of cadmium. At high level treatments, the percentage phytotoxicity of roots and shoots was markedly increased, reported by Naba Kumar Mondal *et al.* (2013).

### 2.3.5. Effect of cadmium on metal tolerance index

As the cadmium concentration prolific the tolerance of heavy metal in plant decreases many reports are available on metal hardship in various plants. Tolerance index decreases in *Triticum aestivum*, reported by Isak Rajjak Shaikh *et al.* (2013). The tolerance of germinated seedlings of *C. maxima* shows adverse effects at all concentrations of $Cd^{2+}$. Highest percentage of tolerance was recorded at 25 ppm (94.07%) of $Cd^{2+}$ whereas, the lowest tolerance key was recorded in 500 ppm with an average of 17.21%, when compared with control, reported by Subin and Steffy Francis (2013).

## 2.4. Effect of cadmium on Biochemical parameters

Heavy metals brought a considerable decrease in the rate of all the metabolic phenomenon of the cell including nucleic acid synthesis, cell division and protein contents (Siegel, 1977; Maitra and Mukherji, 1976; Wickliff *et al.*1980; Nag *et al*., 1984). All biochemical parameters show noticeable variation due to the toxic outcome of cadmium.

### 2.4.1. Effect of cadmium on total protein content

Protein content in organisms is an important indicator of reversible and irreversible changes in metabolism, was known to respond to a wide variety of stressors such as natural and xenobiotic (Singh and Tewari, 2003). Several workers contributed on the effect of heavy metals on protein content in plants. It was

significantly reduced in the cereal wheat by the increasing dose of heavy metal (Tandon and Gupta, 2002). Metal induced inhibition of protein synthesis was also earlier reported by Samantary (2000). Reduction in protein content was observed by the different doses of cadmium in *Cajanus cajan* and *Trigonella foenum-graecum*. Such reduction in protein contents due to the treatment of cadmium and zinc was reported by other workers also. Singh and Tewari (2003) and Liu *et al*. (2005) found that cadmium resulted in a significant inhibition of protein level in *Brassica juncea* L. and in barely seedling. The decrease in protein under the stress of cadmium and lead in *Vigna* and Hydrilla plants was reported by Bhattacharya and Choudhari (1994), such decrease in protein content under the heavy metal stress was also reported by Vyas and Puranik (1993). Chaoui *et al.* (1997) reported decrease in protein content in *Phaseolus vulgaris* roots by the treatment of cadmium and zinc. There was significant decrease in protein content in the leaves of *Artemisia annua* (Khudsar *et al*., 2004).

### 2.4.2. Effect of cadmium on total sugar content

Total sugar content reduces in plants by the different doses of cadmium. The other workers also observed decline in sugar contents due to the effect of cadmium, zinc and other heavy metals. Decrease in soluble sugar content by the treatment of cadmium was reported by Huang *et al*. (2006) in rice seedling. The presence of cadmium in the growth medium of rice plants caused marked perturbations in sugar metabolism. Such events might impair carbohydrate metabolism ultimately leading to impaired growth in seedling (Verma and Dubey, 2001). Singh *et al*. (2007) observed that the supra optimal copper concentrations in leaf tissue of wheat significantly reduced the total sugar content at $14^{th}$ and $21^{st}$ day of treatment. Decrease in sugar content by the treatment of heavy metal in wheat seedling was reported by Tandon and Gupta (2002). The carbohydrate concentration in sugar beet plants has been shown to reduce when cadmium is present in the culture medium (Greger and Lindberg, 1986).

### 2.4.3. Effect of cadmium on proline content

A common response of plants to environmental stress is an accumulation of certain specific metabolites such as amino acids, organic acids and phytochelatins.

Proline, which occurs widely in higher plants, accumulates in larger amounts than other amino acids under heavy metal, salt or water stress. However, according to the contrasting reports on the role of proline in metal tolerance, its use as selection criterion for salt tolerance has been questioned (Ashraf and Harris, 2004). Zengin and Kirbag, 2007 reported increase in proline content of sunflower plant under heavy metal stress. Accumulation of proline and free amino acid in the tissue seems to be wide-spread among plants in response to heavy metal toxicity (Alia and Saradhi, 1991; Bassi and Sharma, 1993; Schat *et al*., 1997; Thomas *et al*., 1998; Shukla *et al*., 2002; Hedaya, 2008). Proline increased in *Cajanus cajan*, *Vigna mungo* and *Triticum aestivum* cultivars under heavy metal stress (Ali and Saradhi, 1991). However, from study of sorghum genotypes Zaifnejad *et al*. (1997) reported that proline did not increase in shoots and roots of plants grown with aluminum.

Proline metabolism is a typical biochemical adaptation in living organisms subjected to stress conditions (Delauney and Verma, 1993). Accumulation of proline in response to cadmium treatment has been previously found in *Solanum nigrum* (Jin *et al*., 2009). Increase in proline concentration has also been reported after treatment of plants with nickel (Gajewska and Sklodowska, 2005), chromium (Pandey and Sharma, 2002) and zinc (Alia *et al*., 1995).

**3.1 Effect of Sodium Nitroprusside on different enzymatic activity**

Various authors working on different plant species that an activity of SOD, APX, GR, CAT and POD increases under salinity stress (Ahmad et al. 2010; Koyro et al. 2012). Rise in activity of enzymatic antioxidants is a protective reaction of plants in order to prevent damage to cellular components due to overproduction of ROS under saline conditions, and can improve salt tolerance by scavenging of ROS (Alscher et al. 2002).. Exogenous application of NO increased activity of CAT, SOD, POD and APX in seashore mallow (Guo et al. 2009), mustard (Zeng et al. 2011), wheat (Ruan et al. 2002), chickpea (Sheokand et al. 2010), and protected plants from oxidative damage under salt stress. Root pre-treatment with NO increased the activity of SOD, CAT, APX and GR, promoted maintenance of cellular redox homeostasis and mitigated oxidative damage under saline conditions in bitter orange (Citrus

aurantium L.) (Tanou et al. 2009). Similarly, exogenous NO increased the activity of antioxidant enzymes (SOD, CAT, and APX) in rice, thus increasing its resistance for salinity (Uchida et al. 2002). In tomato, exogenous application of NO increased the activity of antioxidant enzymes SOD, POD, CAT, APX, non-enzymatic antioxidant ascorbate and reduced glutathione under salinity stress thus helping to alleviate salt-induced oxidative damage (Wu et al. 2011). Several osmolytes, including glycine betaine, sugar alcohols, soluble sugars, proline, trehalose, polyols, etc. have been reported to accumulate in various plant species under salinity and drought (Yancey et al. 1982; Bohnert et al. 1995; Hasegawa et al. 2000; Farooq et al. 2009). In addition to their role in the maintenance of water balance in plant tissues, these osmolytes also act as osmoprotectants; for instance, proline scavenges free radicals (Chen & Murata 2011). NO stimulates cytosolic synthesis of proline and glycine betaine. For example, exogenous application of SNP significantly increased cytosolic proline accumulation in seashore mallow (Kosteletzkya virginica L.), conferring salinity resistance (Guo et al. 2009). Moreover, exogenous NO increased proline accumulation in wheat, where it scavenges ROS and stabilises the structure of the macromolecules (Ruan et al. 2002).

### 3.2 Importance application of SNP (NO) in alleviating salt stress

Pea cultivars differ in their salt compassion (Karlidag et al. 2009) and one of the reason liable for these differences might be their antioxidant status (Hasanuzzaman et al. 2012). Plants with higher activity of enzymatic and non-enzymatic antioxidants can fight ROS and/or oxidative damage more effectively.. Besides of the antioxidative effect of NO (Beligni et al. 2002), this compound can lead to reduction in Na/K ratio in shoots and roots (our study, data not shown) what additionally increases plants tolerance for saline environment. According to Farooq et al. (2009) NO regulates strategies responsible for salinity resistance. When this signalling molecule reaches a plant before initiation of stress, it triggers reactions which lead to increase in leaves antioxidants bustle and higher potential for K assimilation under salinity stress, as a result the plant become more salinity tolerant before $CdCl_2$ comes to play. So, when plants are pre-treated with NO, they become pre-conditioned to better tolerance to the salt stress. This is one of the important

factors of the higher yield, shoot and root fresh and dry weigh (data not shown) in plants pre-treated with SNP in assessment to plants treated with SNP after the salt stress initiation. Exogenous application of NO after initiation of stress can also be helpful, but as some salt-induced damages might exchange to irretrievable form, plant must expend more energy and resources for damages reimbursement or recovery. Pre-treatment or at least, NO application at early phases of stress seems a better strategy for protection because plants may avoid the stress effects or tolerate it better.

Plants frequently respond to stressors by reprogramming their physiological processes to ensure the maintenance of homeostasis and the cell functions. Processes such as photosynthesis, photorespiration, and respiration are basically related and essential to the plant's tolerance to abiotic stresses, being vigilantly regulated in unfavourable conditions (Millar et al., 2011; Gupta et al., 2014; Osakabe et al., 2014). This regulation involves both changes in the cellular redox and cell signaling, with the participation of various secondary messengers, such as NO (Millar et al., 2011).

Nitric oxide is a cell signaling molecule mainly important in the plant's tolerance to stress. In the last few years, several expermintal studies showed that NO is able to perk up plant tolerance to As (Shukla et al., 2015; Silveira et al., 2015; Andrade et al., 2016). Strong evidences indicate that NO acts as a second messenger, triggering different cellular responses, such as the increase in antioxidant defense systems (Fan et al., 2014). This molecule also acts as a signal in the major physiological process in the plant, promoting, for example, changes in photosynthesis and respiration under several ecological conditions (Farnese et al., 2016). However, most of these studies gauge the physiological process in a character way, without allowing for that they occur at the same time in the plant cell. As a result, it is still not clear if NO is able to simultaneously alter different physiological processes and therefore reprogram the plant metabolism in order to increase plant tolerance to As. In addition, few studies focus the response of plants to heavy metals and the consequence of NO in cellular organelles. In this way, there are few data that enforces the complementarity between structure and function, which limits the comprehension of tolerance mechanisms. Seeking to fill this gap, the aim of this study was to evaluate the effects of As and NO on the physiology, morphology and ultrastructure of *Pistia*

*stratiotes*, an aquatic macrophyte able to absorb and accumulate large amounts of As (Farnese et al., 2013) and recommended for remediation of surface waters (Lu et al., 2011). We hypothesized that NO would be able to improve the plant tolerance to As by reprogramming the physiological process, which would result in the attenuation of the cellular damages caused by the pollutant.

The decrease triggered by As in $\Phi_{CO2}$ as well as in net $CO_2$ assimilation rates is a reflection of the changes in chloroplasts and the consequent reduction in PSII efficiency, since changes were not observed in $g_s$ and $C_i$ (Xing et al., 2013). The reduction in fixation of $CO_2$ may have occurred also because of the decrease in gene expression of Rubisco and other enzymes of the Calvin cycle in response to the pollutant (Finnegan and Chen, 2012). However, the photosynthetic responses of plants to As appears to be variable among the species. Indeed, while in some plants the photosynthetic limitations triggered by As are essentially biochemical (Srivastava et al., 2013), as observed for *P. stratiotes*, in *Oryza sativa* the decrease in the net carbon assimilation after exposure to the metalloid was mainly a consequence of the stomatal closure (Sanglard et al., 2014). Cadmium, provided separately, promoted increases in plant respiration. This result is in sharp contrast with transcriptional analyses in *Arabidopsis thaliana* exposed to As which indicated a decrease in the enzyme activity of the citric acid cycle (Abercrombie et al., 2008), but the respiratory activity of the plants was not measured. In *P. stratiotes* exposed to the metalloid the increment in respiratory rates were not accompanied by the restoration of cellular homeostasis. In other words, the increase in respiration did not contribute to the restoring of photosynthesis rates, to decrease the ROS concentration or to the maintenance of cell structure. The increased respiration in plants exposed to As is probably a result of the chemical similarity between the phosphate and arsenate, which compete for the same active site of the mitochondrial ATP synthase (Moore et al., 1983). This competition results in the synthesis of a highly unstable product As-ADP, causing a decrease in ATP concentration. Low ATP levels are a signal that triggers the increment in respiratory activity, which leads to the generation of more ADP-As. Therefore, although increases in respiration are important in stress conditions to provide carbon skeletons (Dutilleul et al., 2003), the occurrence of futile

cycles of As-ADP generation compromises the energy status of the cell and can contribute to the increase of ROS production (Finnegan and Chen, 2012). The alterations in cell respiration of plants subjected only to As probably would not hold for longer periods of exposure, as the metalloid triggered the disruption of mitochondrial membrane, which would compromise the respiratory process.

Besides As, NO also improved the respiration rates in plant cells. Unlike what occurs for As, however, the change mediated by NO in the respiration rates was important to achieve cellular homeostasis as indicated by the PCA analysis. Indeed, increases in respiratory process contribute to the plant tolerance by the generation of carbon skeletons and metabolic energy to support processes involved in responses to stress (Fernie et al., 2004; Bolton, 2009). In addition, the improve of respiration rates may have also contributed to maintaining the structure of organelles, once the adequate energy status of the cell is necessary for the synthesis and restoration of cell membranes (Liu et al., 2016) and to decreased ROS generation (Millar et al., 2011). The mechanisms by which NO mediates the regulation of mitochondrial respiration to protect the cells are not fully understood, but evidence indicates the involvement of multiple pathways, including the increase of the pathway of cytochrome c oxidase and the route of alternative oxidase (Jhanji et al., 2012; Shan et al., 2012).

Unlike what was observed in respiration, *P. stratiotes* treated with As showed decreases in the photorespiration, and this reduction was more expressive when the pollutant was supplied together with SNP. In fact, As+SNP decreased the oxygenation rate of Rubisco, increased the $ETR_C/ETR_O$ ratio, decreased the concentration of glycerate and decreased the glycine/serine ratio, one of the main biochemical markers for photorespiration rate (Novitskaya et al., 2002). All of these parameters indicate a reduction of photorespiration, which could be the consequence of the inactivation of enzymes related to photorespiratory process. Evidences of the inhibitory role of NO on photorespiration have also been observed in *Arabidopsis* (Corpas and Barroso, 2016). Several enzymes involved in photorespiration are targets of S-nitrosylation by NO, as the enzyme glycolate oxidase (GOX), which is inhibited by this process. The enzyme GOX is a key enzyme in photorespiration and its activity results in the formation of $H_2O_2$. Thus, it is believed that NO might be important in the regulation

of the levels of ROS by post-translational modifications of one of the major enzymes responsible for $H_2O_2$ production (Ortega-Galisteo et al., 2012). Such regulation is necessary because, although the photorespiration plays important physiological roles, under stress conditions this process can be responsible for the generation of up to 70% of all cellular $H_2O_2$ and is considered the main source of ROS in plants submitted to As (Gupta et al., 2013).

In addition to the changes in physiological processes in chloroplasts and mitochondria, it should also be considered that the presence of As disrupted all the protoplast of the mesophyll cells of *P. stratiotes.* It is likely that the excess of ROS from plants exposed to As are involved in the disruption of the membrane system, especially in plasma and vacuolar membranes. In the case of the vacuolar membrane, it has been suggested that ROS participate in the cell signaling cascade that culminates in the activation of the vacuolar enzyme of processing, which modifies the structure and causes collapse of the membrane of the vacuole (Li et al., 2013). Additionally, it is likely that the increase in ROS concentration has a direct effect on the denaturation of the cytosol through the oxidation of biomolecules (Jin et al., 2010). In relation to trichomes, the premature senescence was also observed in *Ocimum basilicum* exposed to As and probably is related to the preferential accumulation of the pollutant in these structures (Biwas et al., 2015).

The maintenance of the integrity of some organelles, especially mitochondria, until the cell death, is an evidence of the occurrence of programmed cell death in *P. stratiotes* exposed to As (Schussler and Longstreth, 1996). In this process, the increase in ROS concentration acts as a signal that ultimately changes the pattern of gene expression and triggers cell death (Laloi et al., 2006). Evidence for programmed cell death was not observed in the As + SNP treatment. It is believed that NO is able to retard or prevent this process, which is probably a result of its influence on the concentration of ROS (Beligni et al., 2002)..

It is likely that the excess of ROS from plants exposed to Cd are involved in the disruption of the membrane system, especially in plasma and vacuolar membranes. In the case of the vacuolar membrane, it has been suggested that ROS participate in the cell signaling cascade that culminates in the activation of the

vacuolar enzyme of processing, which modifies the structure and causes collapse of the membrane of the vacuole (Li et al., 2013).

### 3.3 The Antioxidant the Effects of NO Donors on Aleurone Cell Viability

NO has been shown to act synergistically with ROS in plant and animal cells to promote cell death (Delledonne et al., 1998; Wendehenne et al., 2001), but NO has also been shown to act as an antioxidant and to prevent death (Beligni and Lamattina, 1999; Beligni and Lamattina, 2001; Wendehenne et al., 2001).

The upholding of the integrity of some organelles, especially mitochondria, until the cell death, is an evidence of the occurrence of programmed cell death in *P. stratiotes* exposed to As (Schussler and Longstreth, 1996). In this process, the increase in ROS concentration acts as a signal that ultimately changes the pattern of gene expression and triggers cell death (Laloi et al., 2006). In plants subjected to as, the coexistence of cells with distinct levels of structural damage, including cells with normal manifestation, proved to be compatible with the symptoms perceived externally on the leaves The ultra-structural analysis showed that this necrosis was a consequence of membrane vesiculation, obviously causing rupture and cytoplasm leakage. Evidence for programmed cell death was not observed in the Cd + SNP treatment. It is believed that NO is able to retard or prevent this process, which is probably a result of its influence on the concentration of ROS (Beligni et al., 2002).

Besides As, NO also improved the respiration rates in plant cells. Unlike what occurs for Cd, however, the change mediated by NO in the respiration rates was important to achieve cellular homeostasis as indicated by the PCA analysis. Indeed, increases in respiratory process contribute to the plant tolerance by the generation of carbon skeletons and metabolic energy to support processes involved in responses to stress (Fernie et al., 2004; Bolton, 2009). In addition, the improve of respiration rates may have also contributed to maintaining the structure of organelles, once the adequate energy status of the cell is necessary for the synthesis and restoration of cell membranes (Liu et al., 2016) and to decreased ROS generation (Millar et al., 2011). The mechanisms by which NO mediates the regulation of mitochondrial respiration to protect the cells are not fully understood, but evidence indicates the involvement of

multiple pathways, including the increase of the pathway of cytochrome c oxidase and the route of alternative oxidase (Jhanji et al., 2012; Shan et al., 2012).

**NO was observed in respiration regulation in stress**,

*P*ea treated with Cd showed decreases in the photorespiration, and this lessening was more expressive when the pollutant was supplied together with SNP. In fact,Cd +SNP decreased the oxygenation rate of Rubisco, increased the $ETR_C/ETR_O$ ratio, decreased the concentration of glycerate and decreased the glycine/serine ratio, one of the main biochemical markers for photorespiration rate (Novitskaya et al., 2002). Evidences of the inhibitory role of NO on photorespiration have also been observed in *Arabidopsis* (Corpas and Barroso, 2016). Several enzymes involved in photorespiration are targets of S-nitrosylation by NO, as the enzyme glycolate oxidase (GOX), which is inhibited by this process. The enzyme GOX is a key enzyme in photorespiration and its activity results in the formation of $H_2O_2$. Thus, it is believed that NO might be important in the regulation of the levels of ROS by post-translational modifications of one of the major enzymes responsible for $H_2O_2$ production (Ortega-Galisteo et al., 2012).).

**Salicylic acid (SA**)

Salicylic acid (SA) is a phenolic compound (Fig. 1) which, despite its broad distribution in plants, has basal levels differing widely among species, with up to 100-fold differences having been recorded (Raskin *et al.*, 1990). This disparity can be observed within members of the same family. For example, in the Solanaceae, whereas tobacco (*Nicotiana tabacum*) contains low basal levels of SA [<100 ng $g^{-1}$ fresh weight (FW)] in leaves (Yalpani *et al.*, 1991; Malamy *et al.*, 1992), potato (*Solanum tuberosum*) might contain up to 10 μg of total SA $g^{-1}$FW (Coquoz *et al.*, 1998; Navarre and Mayo, 2004). In the model plant *Arabidopsis thaliana*, basal levels of total SA range from 0.250 μg to 1 μg $g^{-1}$ FW (Nawrath and Métraux, 1999; Wildermuth *et al.*, 2001; Brodersen *et al.*, 2005). SA is synthesized through two distinct and compartmentalized pathways that employ different precursors: the phenylpropanoid route in the cytoplasm initiates from phenylalanine, and the isochorismate pathway takes place in the chloroplast. Most of the SA synthesized in

plants is glucosylated and/or methylated. Glucose conjugation at the hydroxyl group of SA results in formation of the SA glucoside [SA 2-*O*-β-D-glucoside] as a major conjugate, whereas glucose conjugation at the SA carboxyl group produces the SA glucose ester in minor amounts.

Raskin, 1999; Song, 2006). SAG is actively transported from the cytosol into the vacuole of soybean and tobacco cells, where it may function as an inactive storage form that can release free SA (Dean and Mills, 2004; Dean *et al.*, 2005). Interestingly, SA is also converted to methyl salicylate (MeSA) by an SA carboxyl methyltransferase, and this volatile derivate is an important long-distance signal in tobacco and *Arabidopsis* systemic acquired resistance (Shulaev *et al.,* 1997; Chen *et al.,* 2003; Park *et al.,* 2007; Vlot *et al.,* 2008). MeSA can be further glucosylated to produce MeSA 2-*O*-β-D-glucose, but this SA-conjugated form is not stored in the vacuole (Dean *et al.,* 2005). The reader is referred to excellent reviews dealing with the enzymes and regulation of these biosynthetic routes (Klessig and Malamy, 1994; Lee *et al.*, 1995; Shah, 2003; Chen *et al.*, 2009; Vlot *et al.*, 2009).

The focus of this review is on the activity of SA in plant growth and development as there is evidence that this hormone regulates processes such as seed germination, vegetative growth, photosynthesis, respiration, thermogenesis, flower formation, seed production, senescence, and a type of cell demise that is not associated with the hypersensitive response. In addition, SA could contribute to maintaining cellular redox homeostasis through the regulation of antioxidant enzymes activity (Durner and Klessig, 1995, 1996; Slaymaker *et al.*, 2002) and induction of the alternative respiratory pathway (Moore *et al.*, 2002), and to regulating gene expression by inducing an RNA-dependent RNA polymerase that is important for post-transcriptional gene silencing (Xie *et al.*, 2001).

**SA enhances germination under abiotic stress:**

When low doses are applied on the surface, SA significantly improves *Arabidopsis* seed germination and seedling establishment under different abiotic stress conditions (Rajjou *et al.*, 2006; Alonso-Ramírez *et al.*, 2009). Under salt

stress (100–150 mM NaCl) only 50% of *Arabidopsis* seeds germinate, but in the presence of SA (0.05–0.5 mM) seed germination increases to 80%. External application of SA also partially reverses the inhibitory effect of oxidative (0.5 mM paraquat) and heat stress (50 °C for 3 h) on seed germination (Alonso-Ramírez *et al.*, 2009). These explanation are in agreement with the delayed germination phenotype observed in the *Arabidopsis sid2* mutant under high salinity (Alonso-Ramírez *et al.*, 2009). This mutant is affected in the isochorismate synthase gene and thus contains low SA levels However, *NahG* transgenic lines expressing a bacterial salicylate hydroxylase also have lower SA levels than wild-type plants, but germination is not affected by high salinity (Borsani *et al.*, 2001). This apparent discrepancy is due to the antioxidant effect of catechol, the product of the salicylate hydroxylase that accumulates in the *NahG* seeds and seedlings (Lee *et al.*, 2010). Thus the germination promotion effect of SA under high salinity condition is by reducing oxidative damage. Moreover, proteomic analyses showed that two superoxide dismutases are promoted by SA

**SA cross-talk with ABA and GAs in case of germination:**

During near the beginning developmental stage, a complex interaction between SA and both ABA and GAs determines germination outcome. In *Arabidopsis*, GAs has a activity in SA biosynthesis and the SA pathway. Imbibition of 50 μM $GA_3$ by seeds for 24 h, as well as the over expression of a GA-stimulated gene from beechnut (*$FcGASA_4$*) in *Arabidopsis* plants, induces a 2-fold increase in SA levels compared with seeds imbibed in water and wild-type plants. Furthermore, increased expression of the *ICS1* (*isochorismate synthase*) and *NPR1* (*nonexpressor of PR-1*) genes, involved in SA biosynthesis and perception, respectively, is observed in *$FcGASA_4$*-overexpressing lines, and in Col-0 seedlings grown in the presence of $GA_3$. Interestingly, external SA (50 μM) partially to save seed germination in the GA-deficient mutant *ga1-3*, whereas external $GA_3$ (50 μM) slightly better the germination of the SA-deficient *sid2* mutants under 150 mM NaCl stress (Alonso-Ramírez *et al.*, 2009). Although these results suggest a synergistic relationship between SA and GA, an antagonistic relationship was observed during barley germination that could be

explained by the addition of a higher dose of SA. The inhibition of barley seed germination and post-germination growth by SA is accompanied by suppression of GA-induced α-amylase (*Amy32b*) expression through initiation of a WRKY repressor (HvWRKY38). Appearance of *HvWRKY38* in aleurone cells is down-regulated by GAs, but up-regulated by SA and ABA, so this transcription factor might serve as a converging node of the SA and ABA signal pathways drawn in suppressing GA-induced seed germination (Xie *et al.*, 2007).

**4. Seed priming**

Seed priming was first anticipated by Heydecker (1973). Seed priming is an effective technology to enhance rapid and uniform emergence and to achieve high vigour, leading to better stand establishment and yield. It is a simple and low cost hydration technique in which seeds are partially hydrated to a point where pre-germination metabolic activities start without actual germination, and then re-dried until close to the original dry weight. Seed priming is employed for better crop stand and higher yields in a range of crops. Harris et al., (2007) reported that seed priming led to better establishment and growth, earlier flowering, increase seed tolerance to adverse environment and greater yield in maize. The valuable effects of seed priming have been demonstrated for many

**4.1 Seed priming effects on germination**:

Stand abolition is of most importance for maximizing field production of any crop plant. At suboptimal conditions of environment conditions, poor seed germination and subsequently poor field is a common abolition phenomenon. It has been reported that one of the major impedes to high yield and production of crop plants is the lack of synchronized crop establishment due to poor weather and soil conditions (Mwale et al., 2003). On the other hand seeds are occasionally sown in seedbeds having adverse moisture because of the lack of rainfall at sowing time which results in poor and unsynchronized seedling emergence (Angadi and Entz, 2002). Strategies for improving the growth and development of crop species have been investigated for many years. Quick germination and emergence are essential for successful crop establishment, field crops such as wheat, sweet corn, mung bean,

barley, lentil, cucumber etc. (Sadeghian and Yavari, 2004). Rehman et al., (2011) reported that seed priming is a cost effective technology that can enhance early crop growth leading to earlier and more uniform stand with yield associated benefits in many field crops including oilseeds.

**4.2 Effect of priming intensity**

The probable reason could be that priming of seeds results in an increased seedling vigor and strength and more established root growth, which enhanced the plant competency for light, water and nutrients resulting in more established plants. Seed priming increase cell division and seedling roots which cause an increase in plant height (Singh et al., 2015). Kumar et al., (2002) reported that 8 hours priming of finger millet seeds in water resulted in an increased mean plant height. Similar results regarding plant height due to the seed priming with Zn solution were reported by Arif et al., (2005) and Ali et al., (2005). These results confirmed the findings reported by Rashid et al., (2002), who illustrated that seed priming improves the plant growth and stand. Moreover, Asgedom and Becker (2001) monitored that P and Zn primed seeds showed higher vigor than unprimed seed as reflected in maximum plant height. Alam et al., (2013) also reported.

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*Chapter III*

# MATERIALS AND METHODS

A experiment was carried out in the Plant growth chamber of Department of Plant Physiology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi taking field pea genotypes cons wasting with five treatments and three replications. The materials used and the methods employed are given in this chapter

## 3.1 Experimental site

The experimental was conducted in the Plant growth chamber in our Department (Department of Plant Physiology), Institute of Agricultural Sciences, Banaras Hindu University.

## 3.2 Weather and climate

Temperature required for their growth was set as 25°C day temperature and night temperature was set as 20°C. Light duration was set at 16 hour and night duration was set as 8 hour and humidity was set at 68%

## 3.3 Description of the experiment

Present investigation was carried out taking one genotypes of field pea (HUDP-15). Seeds were procured from the Department of Genetics& Plant Breeding, Institute of Agricultural Sciences; B.H.U., Varanasi. Experiments were carried out as follows

### 3.3.1 Source of experimental material

Disease free, healthy seeds of field pea variety HUDP-15 were procured from Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, BHU, Varanasi. Bold and uniform seeds were selected where as small, discolored, infected seeds were discarded.

### 3.3.2 Varietal details

**a) HUDP-15:** protein rich popular variety

**Year of release**: 2006

**Average grain yield:** 60 q/ha

**Recommended area**: U.P., Bundhelkhand and M.P. particularly of drier zone because it is serving dual purpose (vegetable and seed).

**Maturity**: Late (126 days)

### 3.3.3 Seed sterilization and treatment

Homogenous seeds of genotypes of pea were surface sterilized in $HgCl_2$ solution (0.01%) for 5 minutes to eliminate possible seed borne microorganisms and rinsed three times with sterile distilled water.

### 3.3.4 Germination test

All petriplates and filter papers were disinfected at 120ºC for 2 hours in an oven. Whatsman paper No.1 was placed in petriplates and moistened with distilled water. Ten seeds were placed in three replications for the conduct of germination test. Petri dishes were then covered by placing another Petridis and placed in incubator at 22ºC with 70% relative humidity.

### 3.3.5 Sterilization and Pot filling:

Soil was collected from Experimental Farm, Institute of Agricultural Sciences, Banaras Hindu University. It was cleaned by removing the stones; weeds etc. and the soil to be used in the pots were dried, powdered and mixed thoroughly. Soil and Sand were mixed in the ratio of 2:1 .The pots were washed with tap water and then sterilized by using 70% methanol and kept for drying. The pot filling was done after 5-6 days of soil (treated with 50µM $CdCl_2$ solution) and pot sterilization. Each plastic pot with capacity of containing 2 kg soil and with closed bottom end was filled-up with air dried soil and sand. The outlet present in the bottom of the plastic pots was regulated in such a way that they drain out the excess of water or solution applied to the plants.

### 3.3.6 Seed priming

Before sowing the seeds, the seeds were primed with such as one seed; pot was primed with salicylic acid (SA) of 2mM solution, another pot was primed with sodium nitroprusside (SNP) of 100µM and another pot was primed with salicylic acid(SA) and sodium nitroprusside (SA+SNP) solution.

### 3.3.7 Seed sowing

Surface-sterilized seeds, four in number were sown in each pot. Two healthy plants were maintained after emergence in each pot. The depth of sowing was at 5 cm. Sowing was done on 13 March 2018

### Imposition of heavy metal stress

Plants were exposed to heavy metal stress ($CdCl_2$; 50mM) by soil application one week prior to sowing of seeds.

### Preparation of solutions

(A) Preparation of 2mM salicylic acid (SA)
(B) Preparation of 100µM sodium nitroprusside (SNP)
(C) Preparation of $CdCl_2$; 50mM solution

### Layout and treatment details:

| | **Genotype** | **Symbol Used** |
|---|---|---|
| **A:** | HUDP-15 | G |
| **B:** | | |
| **S.No:** | **Treatment** | |
| 1 | Non primed seeds were sown in un treated soil | $T_0$ |
| 2 | Non primed seeds were sown in $CdCl_2$ treated soil | $T_1$ |
| 3 | SA primed seeds were sown in $CdCl_2$ treated soil | $T_2$ |
| 4 | SNP primed seeds were sown in $CdCl_2$ treated soil | $T_3$ |
| 5 | SA+SNP primed seeds were sown in $CdCl_2$ treated soil | $T_4$ |

1) Treatments 5

2) Varieties: 1

3) Replications: 3

4) Treatment combinations: 5×3×1=15

5) Design: Factorial CRD

**3.5. Sampling procedure and observations**

Physiological and biochemical observations were recorded at 40 and 60 days after emergence. All the samples for biochemical estimation were taken between 8 A.M. to 9 A.M. in the morning. Leaf samples were collected from top 2$^{nd}$ and 3$^{rd}$ leaf and mixed to make final weight of required quantity of sample for estimation.

**3.5.1 Morphological parameters**:

**Germination percentage (%)**

Number of germinated seeds out of total seeds taken in petri dishes in growth chamber was counted at 7 days after sowing (DAS).

**Plant Height (cm):**

Plant height of two plants were taken from the base of the plant to the growing tip of main shoot with the help of meter scale, averaged and expressed in centimeter.

**Number of leaves Plant$^{-1}$ (cm):**

The numbers of leaves plant were counted separately at 40 and 60 DAS in plant growth chamber in controlled and treated plant.

**Leaf area (cm$^2$ plant$^{-1}$)**

The total leaf area of all the counted leaves was measured with the help of leaf area meter (Systronics Leaf Area Meter 211) at 40 and 60 DAS.

### 3.5.2 Physiological and Biochemical Parameters

#### 3.5.2.1 Chlorophyll 'a' and 'b' content and carotenoid content (mg $g^{-1}$ F.W.):

Chlorophyll 'a' and 'b' content and carotenoid content in leaves were determined by the method of Arnon, 1949. The chlorophyll extraction was done in 80% acetone. Leaves were washed thoroughly and dried, cut into small discs. 100 mg of such leaves were placed in test tubes containing 10 mL acetone and chlorophyll was extracted. The suspension was centrifuged at 5000 rpm for 5 minutes and supernatant was used for measuring chlorophyll content .The absorbance was read at 645 nm and 663 nm. Blank was taken to be 80% acetone.

The Chlorophyll content was estimated by the formulae as given below.

Chlorophyll 'a' Content = $(12.7\ X\ A663 - 2.69\ X\ A645)X\ \frac{V}{1000X\ W}$

Chlorophyll 'b' content = $(22.9\ X\ A645 - 4.68\ X\ A663)X\ \frac{V}{1000X\ W}$

Carotenoid content = $4.49\times A_{450}$ -0.26 $(2.02\times A_{645} + 8.02\times A_{663}) \times \frac{V}{1000X\ W}$

#### 3.5.2.2 Ascorbate peroxidase (1.11.1.11), (U enzyme $g^{-1}$ F.W. $min^{-1}$):

Ascorbate peroxidase activity was assayed in leaf samples at 40 days after emergence (DAE) and 60 DAE (to study temporal variation) in normal and cadmium stressed plants. Ascorbate peroxidase was assayed as per the protocol of Nakano and Asada, 1981.

**Reagents:**

**a)** Ascorbic acid (3.0 mM)

**b)** EDTA (3.0 mM)

**c)** Hydrogen peroxide (3.0 mM)

**d)** Phosphate buffer (100 mM, pH 7.0)

**Solution A:** Potassium dihydrogen phosphate (6.80 g) was dissolved in water and the volume was made up to 500 mL with double distilled water.

**Solution B:** Di-potassium hydrogen phosphate (8.71 g) was dissolved in water and the volume was made up to 500 mL with double distilled water.

Phosphate buffer (100 mM, pH 7.0) was prepared by mixing 39 mL of solutin A and 61 mL of solution B, and final pH was adjusted with the help of pH meter.

**Procedure:**

Enzyme extract for APX was prepared by grinding 100 mg sample with 1.0 mL of extraction buffer (0.1 M phosphate buffer, pH 7.5 containing 0.5 mM EDTA and 1 mM ascorbic acid). The homogenate was centrifuged in centrifuge at 10,000 x g for 10 minutes at 4ºC. After centrifugation, supernatant was collected and this supernatant was used as enzyme source.

The 3 mL reaction mixture contained:

50 mM potassium phosphate buffer (pH 7.0) (1.5 mL of 100 mM)

0.5 mM ascorbic acid (0.5 mL of 3.0 mM)

0.1 mM EDTA (0.1 mL of 3.0 mM)

0.1 mM $H_2O_2$ (0.1 mL of 3.0 mM)

0.2 mL enzyme

0.6 mL water (to make a final volume of 3.0 mL).

The reaction was started with the addition of 0.2 mL of hydrogen peroxide. Decrease in absorbance for a period of 30 second was measured at 290 nm in an UV-Visible spectrophotometer Enzyme activity was calculated in terms of amount of ascorbic acid oxidized (initial absorbance – final absorbance = quantity of ascorbic acid oxidized) per minute per mg protein

### 3.5.2.3 Malondialdehyde content (n mol $mL^{-1}$)

Malondialdehyde (MDA) content was determined in leaf samples at 10 DAE, 20 DAE and 30 DAE (to study temporal variation) in normal and cadmium stressed plants. The level of lipid peroxidation was estimated as the MDA content, determined according to the method of Heath and Packer, 1968.

**Reagents:**

A) **Trichloroacetic acid (TCA) (0.1 %, w/v):** Trichloroacetic acid solution was prepared by dissolving 0.1 g TCA in water and then the volume was made to 100 mL.

B) **Thiobarbituric acid reagent (TBA)**: 5 % Thiobarbituric acid (TBA) in 20% TCA was prepared by first preparing 20 % (w/v) TCA (20 g TCA in 100 mL distilled water). Five g TBA was dissolved in a small volume of 20 % TCA and then volume was made to 100 mL by 20 % TCA

**Procedure:**

Plant material (100 mg) was homogenized in 5 mL 5% (w/v) Trichloroacetic acid solution. The homogenate was centrifuged at 10,000 x g for 20 minutes and 0.5 mL of the supernatant was added to 1 mL 0.5% (w/v) thiobarbituric acid in 20% TCA. The mixture was heated at 95ºC for 20 min and immediately cooled in ice bath. The samples were then centrifuged at 10,000 x g for 5 minutes. The absorbance was recorded at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of MDA–TBA complex (red pigment) was calculated from the extinction coefficient as 155 $mM^{-1}$ $cm^{-1}$.

$$\text{MDA content (n mol mL}^{-1}) = \{(A_{532}\text{-}A_{600})/155\}\times 10^3$$

### 3.6.1.1 Protein content

100 mg fresh leaves were taken and homogenized in 5.0 mL 1.5% NaCl using chilled pestle and mortar. The extract was centrifuged at 10,000 × g for 20 minutes. Extraction was repeated twice with 2 mL extraction medium. All supernatents were pooled and final volume was made to 10 mL

**Quantification (Bradford method)**

Protein in the crude extract was determined according to the Coomasie Brilliant Blue G-250 dye binding method (Bradford 1976).

**Bradford Reagent**

Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50 mL ethanol, and to it 100 mL O-phosphoric acid (85% w/v) was added. Total volume was made up to 1 L with double distilled water. The solution was filtered through Whatman No. 1 filter paper and stored in dark bottle at room temperature.

**Procedure**

200 µL of extracted protein was taken. 3.0 mL Bradford dye was added and the samples were incubated at 37ºC for 5 minutes in dark. The absorbance was observed at 595 nm. Protein concentration was calculated with the help of standard curve.

Standard was prepared by taking different concentrations of Bovine serum albumin fraction-V. Remaining procedure was same as for the samples. A curve between known protein concentrations and absorbance was prepared.

**3.6.1.2. Preparation of Standard Curve**

A stock solution of bovine serum albumin (BSA) was prepared by dissolving 50 mg of BSA in 50 mL of distilled water. Then 10 mL of this solution was diluted to 50 mL by adding distilled water. From this solution different concentrations of BSA (10, 20, 50, 100, 200 µg) were prepared by appropriate dilutions. 1 mL of this solution was pipette out in separate test tubes. 3 mL of dye was added to test tubes. These were incubated at room temperature for 10 minutes. Absorbance was taken at 595 nm. Absorbance values were plotted against the concentration to get the standard curve.

**Estimation of Hydrogen peroxide (µmolg$^{-1}$fresh weight)**

Hydogen peroxide was determine in first fully expended leaf from the normal and stressed plants. The estimation was done as per the pprotocol of Mukherjee and Choudhary(1983).

Reagents used

a) Sulfuric acid(2 N)

b) Liquid ammonia solution

c) Acetone solution (80%)

d) Titanium reagent

It was prepared as follows:

One gram (g) titanium dioxide and 10 g potassium sulphate were digested in 15mLconcentrated sulphuric acid over a hot late for 4 hr. The digested mixture was diluted to 500-600mL and stirred with a magnetic stirrer cum heater at 70-80 ºC till a clean transparent solution was obtained. This solution was diluted to 1.5 litres and stored in dark brown bottle.

**Procedure**

Leaf sample (0.1g) was homogenised in 10mL acetone. The homogenate was filtered through Whatman No 1 filter paper. In whole of the extract 4 mL of titanium reagent was added followed by 5mL of concentrated ammonium solution to precipate peroxide- titanium complex. The contents were centrifuged for 5 min at 10000 rpm. The supernatant was discarded and precipitate dissolved in 10mL 2N $H_2SO_4$ . It was centrifuged to remove undissolved material and absorbance was recorded at 415 nm against blank (2N $H_2SO_4$). Concentration of $H_2O_2$ was determined using standard curve plotted with known concentration of $H_2O_2$

**Preparation of Standard Curve**

A standard curb of $H_2O_2$ was prepared by preparing 10, 20, 30, 40, 60, 80, and 100 µM dilution of $H_2O_2$ in double distilled water from stock solution. The $H_2O_2$ titanium complex was formed by adding 4mL of titanium reagent and 5mL of concentrated ammonia solution and processed as described for the sample. Absorbance value were plotted against the concentration..

**3.6.1.3 Superoxide Dismutase Activity (EU $mg^{-1}$ protein $min^{-1}$)**

Superoxide dismutase (SOD) activity was assayed in fully expanded leaves at active tillering, flowering and grain filling stage under normal and delayed sown plants by the method of Dhindsa et al (1981).

**3.3.1.4 Reagents**

**a) Pottassium phosphate buffer (0.1M, pH 7.5) :** Solution of potassium dihydrogen phosphate (0.1 M) (Solution A) and dipotassium hydrogen phosphate (0.1M) (Solution B) prepared by dissolving 13.6 g and 17.4 g salts respectively.Solution A and Solution B were mixed in the ratio of 16.84 and the final pH was adjusted with the help of pH metre to 7.5.

**b) L- Methionine (200 mM):** L-methionine (0.298 g) was dissolved in distilled water and the volume made up to 10 ml. (On the basis of 200 mM we can prepare required quantity of this solution)

**c) Nitro-blue tetrazolium (2.25 mM):** Nitro-blue tetrazolium (0.0184 g) was dissolved in distilled water and the volume was made up to 20 ml and kept in air tight vial.

**d) Ethylene diamine tetra acetic acid (3 mM):** EDTA (0.056 g) was dissolved in distilled water and the volume was made up to 50 ml.

**e) Riboflavin (60 µM):** Riboflavin (0.0023 g) was dissolved in distilled water and the volume was made to 100 ml with distilled water and stored in amber colored bottle at 4˚C in refrigerator.

**f) Sodium carbonate (1.5 M):** Sodium carbonate (15.9 g) was dissolved in distilled water and the volume was made up to 100 ml.

### 3.3.1.5 Preparation of Enzyme extract

Enzyme extract for SOD was prepared by first freezing the weighed amount of leaf samples (100 mg) in ice basket to prevent proteolytic activity. The sample was grinded with 1.0 ml of extraction buffer (0.1 M phosphate buffer, pH 7.5 containing 0.5 mM EDTA). The grinded sample was centrifuged in cooling centrifuge machine at 10000 g for 10 minutes (REMI C-24), after centrifugation supernatant was collected and this supernatant was used as enzyme source.

### 3.3.1.6 Assay of SOD activity

3.0 ml of the reaction mixture containing 0.1 ml of 1.5 M sodium carbonate, 0.2 ml of 200 mM NBT,0.1 ml of 3 mM EDTA, 1.5 ml of 100 mM potassium phosphate buffer, 1 ml of distilled water and 0.1 ml of enzyme extract were taken in test tubes in duplicate from each enzyme sample. Two tubes without enzyme extract were taken as control. The reaction was started by adding 0.1 ml riboflavin (60 µM) and placing the tubes below a light source of two 15W fluorescent lamps for 15 minutes. Reaction was stopped by switching off the light and covering the tubes by black cloth. A non irradiated complete mixture that did not develop colour served as blank. Absorbance was recorded at 560 nm by using spectrophotometer (ELICO SL-196). Enzyme units were calculated as follows:

$$\text{Enzyme Unit (EU)} = \text{Enzyme}^{*}_{\text{(light)}} - \frac{(\text{Enzyme}^{\#}_{\text{(light)}} - \text{Enzyme}^{*}_{\text{(dark)}})}{\text{Enzyme}^{*}_{\text{(light /2)}}}$$

* Without enzyme and # with enzyme

The EU was expressed on per g fresh weight basis as well as on the basis of per mg protein (specific activity).

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*Chapter IV*

# RESULTS AND DISCUSSION

The present investigation is carried out to assess the compatibility of salicylic acid and sodium nitroprusside to reduce the stress effect of cadmium chloride in pea. A number of observations were recorded at two stages of crop growth. Find out the effect of salicylic acid and sodium nitroprusside on including certain physiological, biochemical and antioxidant enzyme activity that influence the growth and sustaining plants to combat the metal stress at the early growth stages.

Pea treated with salicylic acid (SA and sodium nitroprusside (SNP) grown under induced cadmium stress in soil culture is critically studied to evaluate the compatibility of SA and SNP on heavy metal (Cd) stress recovery. A number of observations pertaining to biochemical and compositional determinations were recorded at 40DAS and 60 DAS in pot culture and growing in plant growth chamber in Department of Plant Physiology, Banaras Hindu University Varanasi. Only mean value of the following parameters were considered, which have been presented in various tables and figures in this chapter.

**4.1 Seed germination**

Exogenous application of SA and SNP by seed priming has been extensively studied in this experiment, that SA and SNP showed the noticeable effect in reducing the stress caused due to cadmium chloride. They provide good growth and development under cadmium stress condition which promotes elongation of different growing arts of germinating seed, including hypocotyls, epicotyls and in pea seeds. SA priming seeds and application alleviated salt-induced oxidative stress by reducing malondialdehyde (MDA) content and increasing SOD activity. Interestingly, when comparing SA and SNP treated germinating seeds with untreated seeds.

Table 1 Effect of SA and SNP on germination percentage (%) of a field pea (*Pisum sativium* L.) genotype under incused cadmium stress at 7 days after stages

| **Treatment(T)** | **Genotype(G)** **HUDP-15** | |
|---|---|---|
| **$T_0$** | 85.45 | |
| **$T_1$** | 62.95 | |
| **$T_2$** | 71.52 | |
| **$T_3$** | 75.85 | |
| **$T_4$** | 81.75 | |
| | **SEm ±** | **CD1%** |
| | 1.028 | 2.209 |

Control **$T_0$**:[without $CdCl_2$,SA and SNP];
**$T_1$** [$CdCL_2$ 50 mM];
**$T_2$** [SA 2mM+$CdCl_2$50 mM];
**$T_3$** [SNP100µM+$CdCl_2$ 50 mM];
**$T_4$** [SA 2mM + SNP100µM +$CdCl_2$ 50 mM].

Pre sowing treatment with SA and SNP may be employed as PGR to improve plant growth and crop production under heavy metal stress condition. The effect of $CdCl_2$ solution of germination of pea seeds is shown in table 1 that $CdCl_2$ 50mM delayed and significantly reduce the germination percentage of seeds. Under heavy metal stress condition the germination percentage of the pea seeds genotype HUDP-15 is reduced up to 62.95%( lowest germination percentage) due to $CdCl_2$ on normal condition the germination is good which is 82.45%. SA and SNP show the ameliorative effect in stress condition. They increased the germination percentage and also enhance the rate of germination of pea seeds. SA increased the germination percentage up to 71.52 % and SNP increased the germination 75.85% compared to stress condition it showed positive effect on the plant in the stress condition, but seeds which were primed with SA+SNP mixed solution showed germination percentage (81.75%) and more rapid growth in the seedling compared to stressed, SA and SNP primed seeds.

### 4.2 Shoot length (cm)

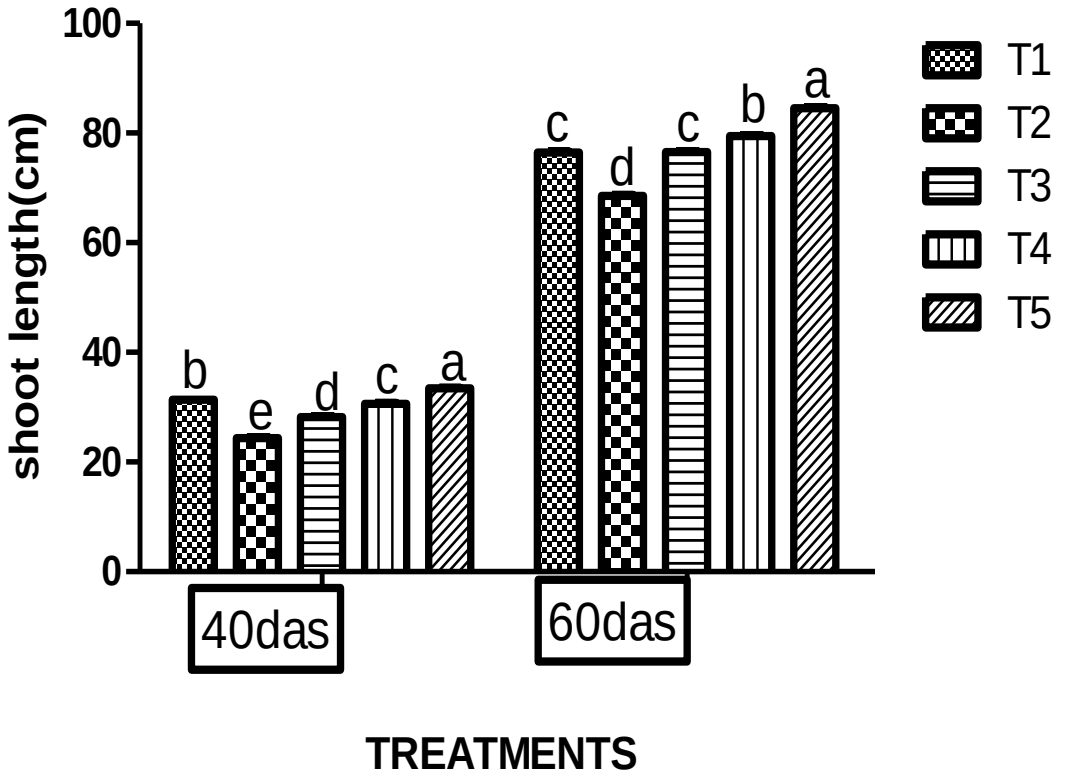
$CdCl_2$ 50mM significantly reduce the mean shoot height. The result obtained from the table is that under heavy metal stress condition without ameliorative primed seeds the shoot height of the pea plant genotype HUDP-15 were reduced by 23.4 % (lowest growth in plant were observed) as compared to control growing plant (were control taken as 100% ). It is observed hear that SA and SNP show the ameliorative effect in stress condition. They increased the germination percentage and also enhance the rate of germination of pea seeds. SA increases the shoot height by 16.75 % and SNP increased the shoot height by 25.71% compared to stress condition it showed positive effect on the plant in the stress condition, but it is observed that those seed which were primed with SA+SNP mixed solution showed more shoot length 38.75% and more vigorous plant compared to stress condition.

Table 2 Effect of salicylic acid (SA) and sodium nitroprusside (SNP) on shoot length plant$^{-1}$ of a field pea (*Pisum sativium* L.) genotype under induced cadmium stress at 40 DAS and 60 DAS interval.

| **Treatment** | **Genotype (G) HUDP-15** | | | |
|---|---|---|---|---|
| | **40 DAS** | | **60 DAS** | |
| **$T_0$** | 31.39 | | 76.40 | |
| **$T_1$** | 24.05 | | 68.80 | |
| **$T_2$** | 28.08 | | 76.42 | |
| **$T_3$** | 30.23 | | 78.86 | |
| **$T_4$** | 33.37 | | 84.23 | |
| | **SEm±** | **CD1%** | **SEm±** | **CD1%** |
| | 0.137 | 0.436 | 0.166 | 0.53 |

Control **$T_0$**:[without $CdCl_2$,SA and SNP];
**$T_1$** [$CdCL_2$ 50 mM];
**$T_2$** [SA 2mM+$CdCl_2$50 mM];
**$T_3$** [SNP100µM+$CdCl_2$ 50 mM]
**$T_4$** [SA 2mM + SNP100µM +$CdCl_2$ 50 mM].

Figure 1 Effect of salicylic acid (SA) and sodium nitroprusside (SNP) on shoot length plant$^{-1}$ of a field pea (*Pisum sativium* L.) genotype under induced cadmium stress at 40 DAS and 60 DAS interval




Control **T$_0$**:[without $CdCl_2$,SA and SNP];
**T$_1$** [$CdCL_2$ 50 mM];
**T$_2$** [SA 2mM+$CdCl_2$50 mM];
**T$_3$** [SNP100µM+$CdCl_2$ 50 mM];
**T$_4$** [SA 2mM + SNP100µM +$CdCl_2$ 50 mM].

### 4.3 Number of leaves plant$^{-1}$

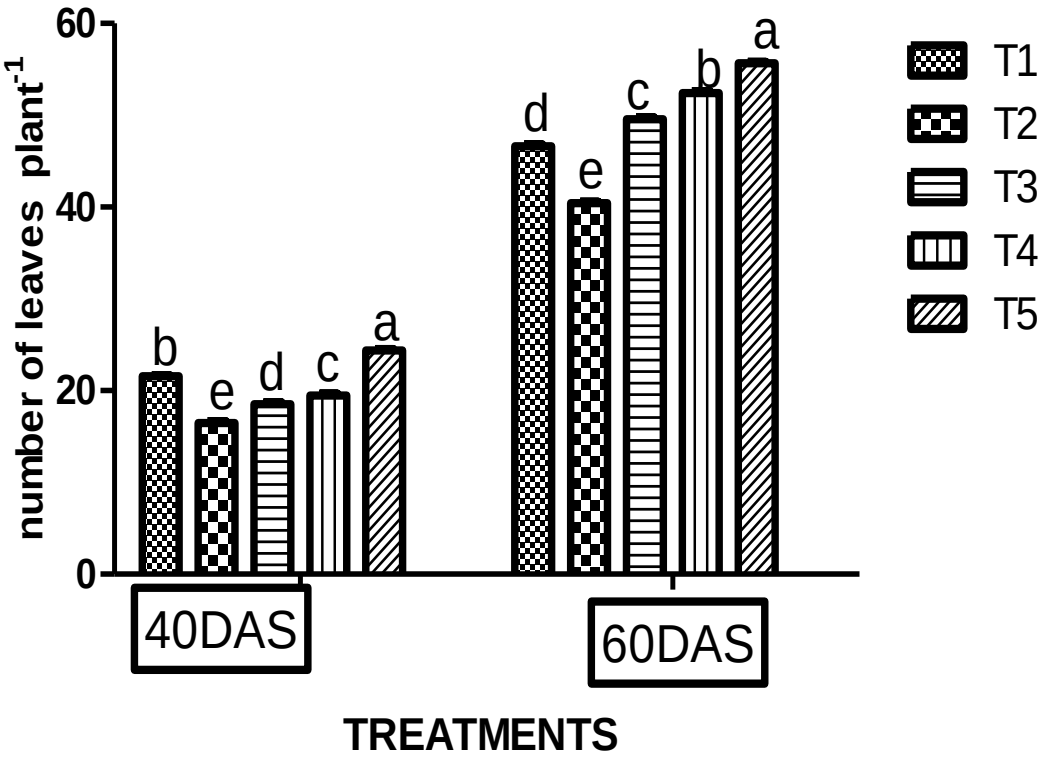
It is observed that number of leaves is increased progressively as the day from sowing . thus the highest 55.73 (mean) number of leaves is found on 60 DAS as compared to 40 DAS, respectively in pots and the minimum16.45 on 40 DAS and 40.19 on DAS number of leaves were observed in $T_1$ which were non ameliorative and exposed to cadmium stress condition. It is observed that maximum number of leaves found in plants whose seeds is treated with SA + SNP solution. SA ameliorative and SNP ameliorative plants nearly the same no of leaves were observed.SA treated plants have 18.34 and SNP treated plants have 19.76 but SNP+SA gives 24.16 on 40 DAS and 55.73 on 60 DAS much more number of leaves plant$^{-1}$.

Table 3 Effect of salicylic acid (SA) and sodium nitroprusside (SNP) on number of leaves plant$^{-1}$ of a field pea (*Pisum sativium* L.) genotype under induced cadmium stress at 40 DAS and 60 DAS interval.

| **Treatment** | **Genotype (G) HUDP-15** | | | |
|---|---|---|---|---|
| | **40 DAS** | | **60 DAS** | |
| **$T_0$** | 21.36 | | 46.23 | |
| **$T_1$** | 16.45 | | 40.19 | |
| **$T_2$** | 18.34 | | 49.13 | |
| **$T_3$** | 19.76 | | 52.31 | |
| **$T_4$** | 24.16 | | 55.73 | |
| | **SEm±** | **CD1%** | **SEm±** | **CD1%** |
| | 0.179 | .572 | 0.186 | 0.895 |

Control **$T_0$**:[without $CdCl_2$,SA and SNP];
**$T_1$** [$CdCL_2$ 50 mM];
**$T_2$** [SA 2mM+$CdCl_2$50 mM];
**$T_3$** [SNP100µM+$CdCl_2$ 50 mM];
**$T_4$** [SA 2mM + SNP100µM +$CdCl_2$ 50 mM].

Figure 2 Effect of salicylic acid (SA) and sodium nitroprusside (SNP) on number of leaves plant$^{-1}$ of a field pea (*Pisum sativium* L.) genotype under induced cadmium stress at 40 DAS and 60 DAS interval.




Control **T₀**:[without $CdCl_2$,SA and SNP];
**T₁** [$CdCL_2$ 50 mM];
**T₂** [SA 2mM+$CdCl_2$50 mM];
**T₃** [SNP100µM+$CdCl_2$ 50 mM];
**T₄** [SA 2mM + SNP100µM +$CdCl_2$ 50 mM].

### 4.4 Leaf area ($cm^2$) $plant^{-1}$

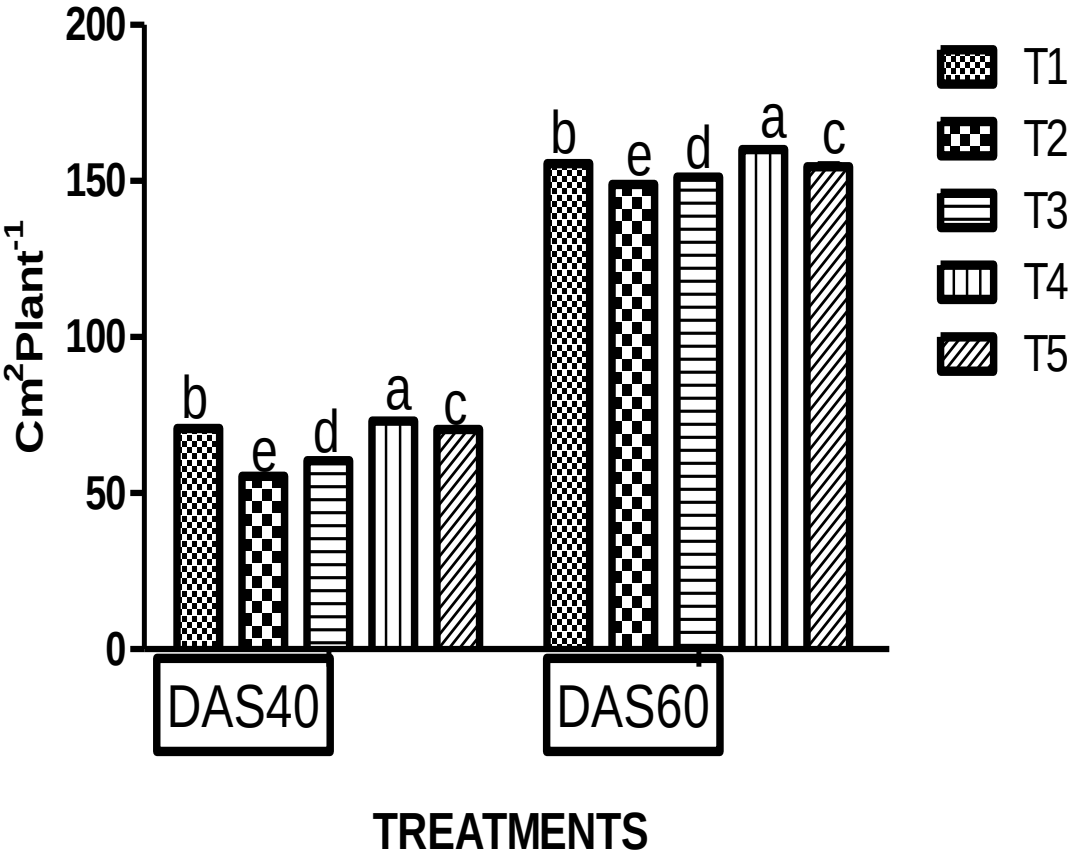
Considerable difference among the various treatments could be observed**.** In this case, the maximum160.11 (mean) $cm^2$ leaves area is found on 60 DAS as compared to 40 DAS, respectively in pots and the minimum 55.40 $cm^2$ on 40 DAS and 148.70 $cm^2$ on 60 DAS area of leaves were observed in $T_1$ which were non ameliorative and exposed to cadmium stress condition Maximum area of leaves found in plants whose seeds is treated with SNP solution. It is found that SA ameliorative and SA+ SNP ameliorative plants have slightly differ in area of leaves were observed.SA treated plants have maximum 151.32 $cm^2$ and SA+ SNP treated plants have maximum154.66 $cm^2$ .

Table 4 Effect of salicylic acid (SA) and sodium nitroprusside (SNP) on leaf area ($cm^2$) $plant^{-1}$ of a field pea (*Pisum sativium* L.) genotype under induced cadmium stress at 40 DAS and 60 DAS interval.

| **Treatment** | **Genotype (G) HUDP-15** | | | |
|---|---|---|---|---|
| | **40 DAS** | | **60 DAS** | |
| **$T_0$** | 70.68 | | 155.60 | |
| **$T_1$** | 55.40 | | 148.70 | |
| **$T_2$** | 60.44 | | 151.32 | |
| **$T_3$** | 73.11 | | 160.11 | |
| **$T_4$** | 70.44 | | 154.66 | |
| | **SEm±** | **CD1%** | **SEm±** | **CD1%** |
| | 1.45 | 2.89 | 1.58 | 3.38 |

Control **$T_0$**:[without $CdCl_2$,SA and SNP];
**$T_1$** [$CdCL_2$ 50 mM];
**$T_2$** [SA 2mM+$CdCl_2$50 mM];
**$T_3$** [SNP100µM+$CdCl_2$ 50 mM];
**$T_4$** [SA 2mM + SNP100µM +$CdCl_2$ 50 mM].

Figure 3 Effect of salicylic acid (SA) and sodium nitroprusside (SNP) on on leaf area ($cm^2$) $plant^{-1}$ of a field pea (*Pisum sativium* L.) genotype under induced cadmium stress at 40 DAS and 60 DAS interval.




Control **T0**:[without $CdCl_2$,SA and SNP];
**T1** [$CdCL_2$ 50 mM];
**T2** [SA 2mM+$CdCl_2$50 mM];
**T3** [SNP100µM+$CdCl_2$ 50 mM];
**T4** [SA 2mM + SNP100µM +$CdCl_2$ 50 mM].

## BIOCHEMICAL ESTIMATIONS

### 4.5 Chlorophyll a content (mgg$^{-1}$ fresh weight)

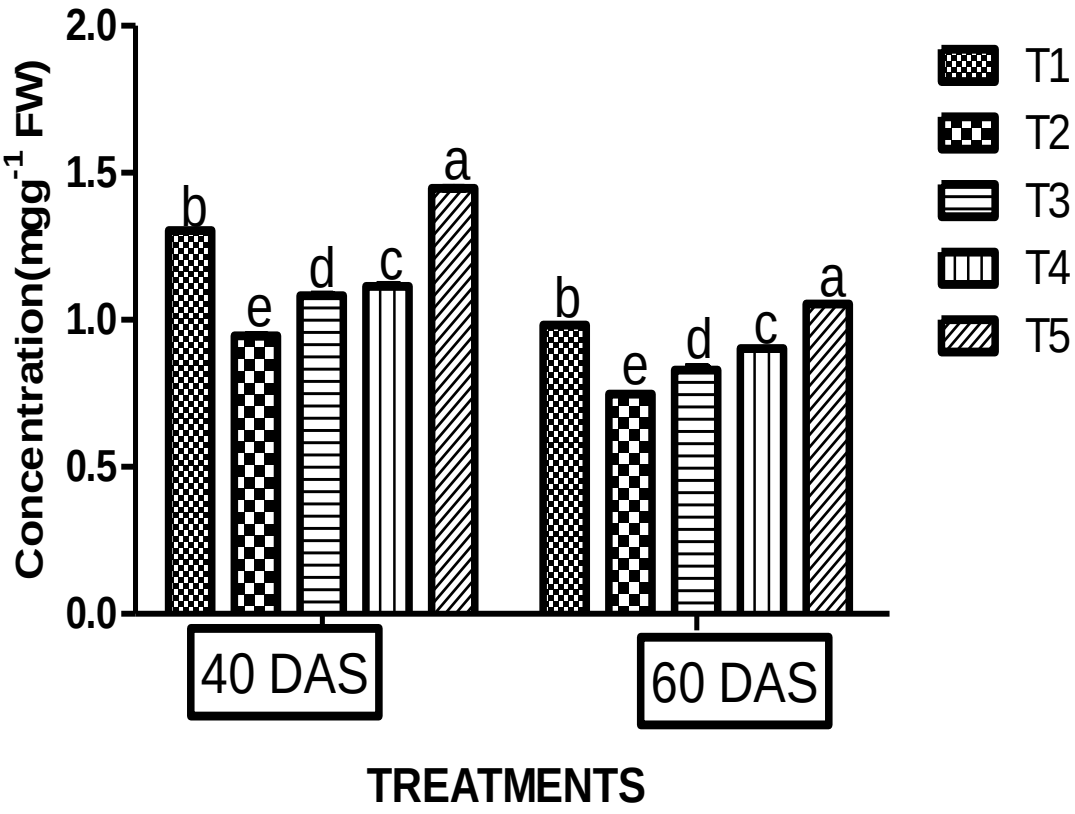
It has already been reported that pea grown under cadmium stress showed reduced photosynthesis**.** In this case, the maximum 1.447 mgg$^{-1}$ fresh weight Chlorophyll a content is found in $T_4$ (SNP +SA) as compared to 1.5 times $T_1$ on 40 DAS and the minimum 0.945 mgg$^{-1}$ fresh weight on 40 DAS and 0.748 mgg$^{-1}$ fresh weight on 60 DAS Chlorophyll a content were observed in $T_1$ which were non ameliorative and exposed to cadmium stress condition. It is observed that 1.087 mgg$^{-1}$Chlorophyll a content found in $T_2$ which is 1.15 times $T_1$ whose seeds were ameliorated with SA solution. It is found that SNP ameliorative plants have slightly more 1.11 mgg$^{-1}$ in Chlorophyll a content which is 1.17 times $T_1$ were observed.

Table 5 Effect of salicylic acid (SA) and sodium nitroprusside (SNP) on Chlorophyll a content of a field pea (*Pisum sativium* L.) genotype under induced cadmium stress at 40 DAS and 60 DAS interval.

| **Treatment** | **Genotype (G) HUDP-15** | | | |
|---|---|---|---|---|
| | **40 DAS** | | **60 DAS** | |
| **$T_0$** | 1.305 | | 0.982 | |
| **$T_1$** | 0.945 | | 0.748 | |
| **$T_2$** | 1.087 | | 0.820 | |
| **$T_3$** | 1.111 | | 0.902 | |
| **$T_4$** | 1.447 | | 0.105 | |
| | **SEm±** | **CD1%** | **SEm±** | **CD1%** |
| | 0.07 | 0.16 | 0.04 | 0.07 |

Control **$T_0$**:[without $CdCl_2$,SA and SNP];
**$T_1$** [$CdCL_2$ 50 mM];
**$T_2$** [SA 2mM+$CdCl_2$50 mM];
**$T_3$** [SNP100µM+$CdCl_2$ 50 mM];
**$T_4$** [SA 2mM + SNP100µM +$CdCl_2$ 50 mM].

Figure 4 Effect of salicylic acid (SA) and sodium nitroprusside (SNP) on Chlorophyll a content of a field pea (*Pisum sativium* L.) genotype under induced cadmium stress at 40 DAS and 60 DAS interval.




Control **T0**:[without $CdCl_2$,SA and SNP];
**T1** [$CdCL_2$ 50 mM];
**T2** [SA 2mM+$CdCl_2$50 mM];
**T3** [SNP100µM+$CdCl_2$ 50 mM];
**T4** [SA 2mM + SNP100µM +$CdCl_2$ 50 mM].

### 4.6 Chlorophyll b content (mgg$^{-1}$ fresh weight)

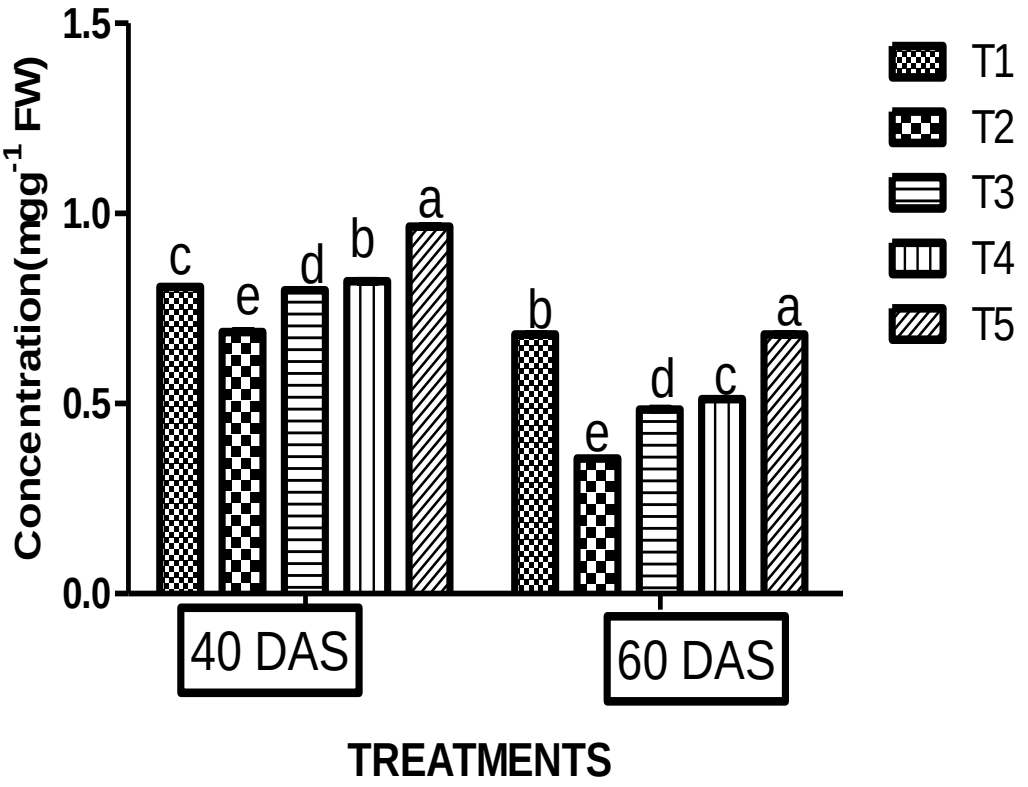
It is considered from table 6 and figure 5 that difference between the various treatments could be distinguished**.** The maximum 0.956 mgg$^{-1}$ fresh weight Chlorophyll b content is found in $T_4$ (SNP +SA) as compared to 1.4 times $T_1$ on 40 DAS and the minimum 0.689 mgg$^{-1}$ fresh weight on 40 DAS and 0.748 mgg$^{-1}$ fresh weight on 60 DAS Chlorophyll b content were observed in $T_1$ ,which were non ameliorative and exposed to cadmium stress condition. It is observed that 0.798 mgg$^{-1}$Chlorophyll content found in $T_2$ which is 1.15 times $T_1$ whose seeds were ameliorated with SA solution. It is found that SNP ameliorative plants have slightly more 1.11 mgg$^{-1}$ in Chlorophyll a content which is 1.19 times $T_1$ were observed.

Table 6 Effect of salicylic acid (SA) and sodium nitroprusside (SNP) on Chlorophyll b content of a field pea (*Pisum sativium* L.) genotype under induced cadmium stress at 40 DAS and 60 DAS interval.

| **Treatment** | **Genotype (G) HUDP-15** | | | |
|---|---|---|---|---|
| | **40 DAS** | | **60 DAS** | |
| **$T_0$** | 0.807 | | 0.681 | |
| **$T_1$** | 0.689 | | 0.356 | |
| **$T_2$** | 0.798 | | 0.485 | |
| **$T_3$** | 0.821 | | 0.512 | |
| **$T_4$** | 0.956 | | 0.681 | |
| | **SEm±** | **CD1%** | **SEm±** | **CD1%** |
| | .05 | .09 | .03 | 0.07 |

Control **$T_0$**:[without $CdCl_2$,SA and SNP];
**$T_1$** [$CdCL_2$ 50 mM];
**$T_2$** [SA 2mM+$CdCl_2$50 mM];
**$T_3$** [SNP100µM+$CdCl_2$ 50 mM];
**$T_4$** [SA 2mM + SNP100µM +$CdCl_2$ 50 mM].

Figure 5 Effect of salicylic acid (SA) and sodium nitroprusside (SNP) on Chlorophyll b content of a field pea (*Pisum sativium* L.) genotype under induced cadmium stress at 40 DAS and 60 DAS interval.




Control **$T_0$**:[without $CdCl_2$,SA and SNP];
**$T_1$** [$CdCL_2$ 50 mM];
**$T_2$** [SA 2mM+$CdCl_2$50 mM];
**$T_3$** [SNP100µM+$CdCl_2$ 50 mM];
**$T_4$** [SA 2mM + SNP100µM +$CdCl_2$ 50 mM].

### 4.7 Total Chlorophyll content (mgg$^{-1}$ fresh weight)

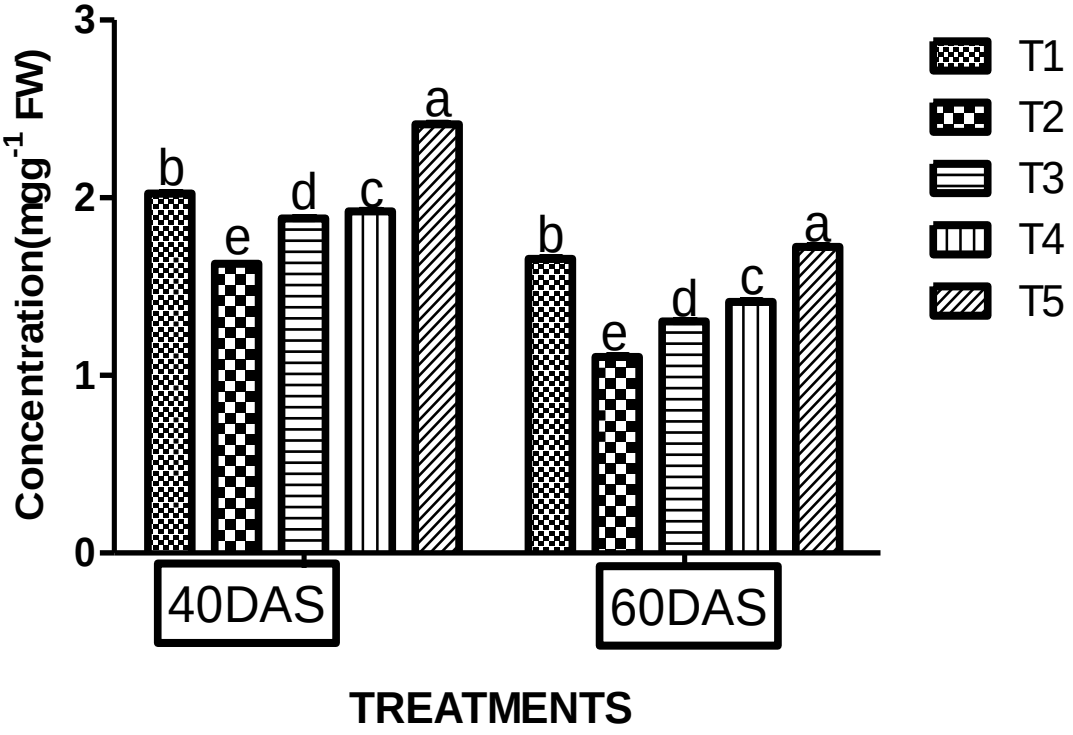
It is considered from table 7 and figure 6 that difference between the total Chlorophyll content from various treatments could be distinguished**.** The maximum 2.41 mgg$^{-1}$ fresh weight total Chlorophyll content is found in $T_4$ (SNP +SA) as compared to 1.47 times $T_1$ on 40 DAS and the minimum 1.63 mgg$^{-1}$ fresh weight on 40 DAS and 1.10 mgg$^{-1}$ fresh weight on 60 DAS total Chlorophyll content were observed in $T_1$ ,which were non ameliorative and exposed to cadmium stress condition. It is observed that 1.89 mgg$^{-1}$ total Chlorophyll content found in $T_2$ which is 1.15 times $T_1$ whose seeds were ameliorated with SA solution. It is found that SNP ameliorative plants have slightly more 1.11 mgg$^{-1}$ in Chlorophyll a content which is 1.18 times $T_1$ were observed.

Table 7 Effect of salicylic acid (SA) and sodium nitroprusside (SNP) on total Chlorophyll content of a field pea (*Pisum sativium* L.) genotype under induced cadmium stress at 40 DAS and 60 DAS interval.

| **Treatment** | **Genotype (G) HUDP-15** | | | |
|---|---|---|---|---|
| | **40 DAS** | | **60 DAS** | |
| **$T_0$** | 2.20 | | 1.66 | |
| **$T_1$** | 1.63 | | 1.10 | |
| **$T_2$** | 1.89 | | 1.30 | |
| **$T_3$** | 1.93 | | 1.41 | |
| **$T_4$** | 2.41 | | 1.73 | |
| | **SEm±** | **CD1%** | **SEm±** | **CD1%** |
| | 0.15 | .08 | 0.04 | 0.04 |

Control **$T_0$**:[without $CdCl_2$,SA and SNP];
**$T_1$** [$CdCL_2$ 50 mM];
**$T_2$** [SA 2mM+$CdCl_2$50 mM];
**$T_3$** [SNP100µM+$CdCl_2$ 50 mM];
**$T_4$** [SA 2mM + SNP100µM +$CdCl_2$ 50 mM].

Figure 6 Effect of salicylic acid (SA) and sodium nitroprusside (SNP) on total Chlorophyll content of a field pea (*Pisum sativium* L.) genotype under induced cadmium stress at 40 DAS and 60 DAS interval.




Control **$T_0$**:[without $CdCl_2$,SA and SNP];
**$T_1$** [$CdCL_2$ 50 mM];
**$T_2$** [SA 2mM+$CdCl_2$50 mM];
**$T_3$** [SNP100µM+$CdCl_2$ 50 mM];
**$T_4$** [SA 2mM + SNP100µM +$CdCl_2$ 50 mM].

### 4.8 Hydrogen peroxide content (µM g-1 fresh weight)

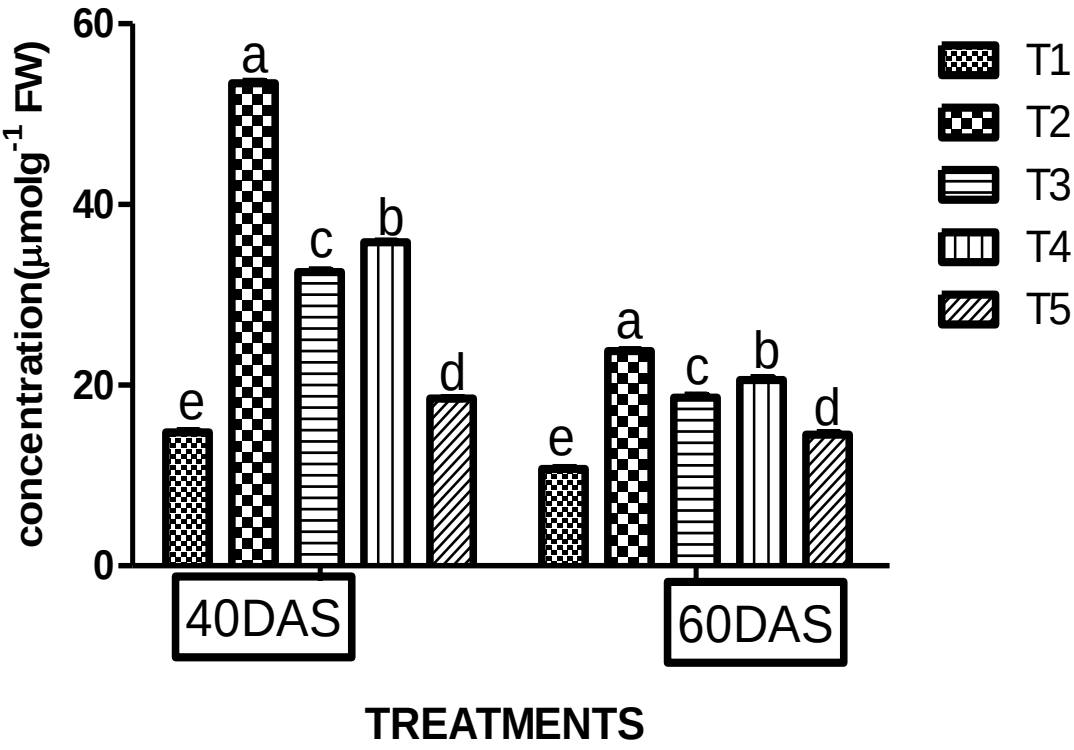
Hydrogen peroxide content in the pea plants is increased under induced cadmium stress. It is observed that minimum $H_2O_2$ content 14.95 µM g-1 fresh weights of leaves found on 40 DAS in plant whose seeds is untreated (control). The maximum $H_2O_2$ content 53.52 µM g-1 fresh weight is found on 40 DAS as compared to other treated plants, respectively in pots( $T_1$) which were non ameliorative and exposed to cadmium stress condition. The value of $H_2O_2$ in $T_1$ is nearly 3.6 times higher than the control ($T_0$) observed on 40 DAS .The $H_2O_2$ value of $T_2$ is 40% lesser than $T_1$. The $H_2O_2$ value of $T_3$ is 33% lesser than T1. The best ameliorative effect were shown by $T_4$ (SA+SNP) which showed 65% lesser $H_2O_2$ value as compared to $T_1$ on the basis of 40 DAS.

Table 8 Effect of salicylic acid (SA) and sodium nitroprusside (SNP) on Hydrogen peroxide content (µM g-1 fresh weight)of a field pea (*Pisum sativium* L.) genotype under induced cadmium stress at 40 DAS and 60 DAS interval.

| **Treatment** | **Genotype (G) HUDP-15** | | | |
|---|---|---|---|---|
| | **40 DAS** | | **60 DAS** | |
| **$T_0$** | 14.95 | | 10.65 | |
| **$T_1$** | 53.52 | | 23.69 | |
| **$T_2$** | 32.65 | | 18.26 | |
| **$T_3$** | 35.89 | | 20.89 | |
| **$T_4$** | 18.45 | | 14.36 | |
| | **SEm±** | **CD1%** | **SEm±** | **CD1%** |
| | 1.06 | 2.25 | 0.30 | 0.62 |

Control **$T_0$**:[without $CdCl_2$,SA and SNP];
**$T_1$** [$CdCL_2$ 50 mM];
**$T_2$** [SA 2mM+$CdCl_2$50 mM];
**$T_3$** [SNP100µM+$CdCl_2$ 50 mM];
**$T_4$** [SA 2mM + SNP100µM +$CdCl_2$ 50 mM].

Figure 7 Effect of salicylic acid (SA) and sodium nitroprusside (SNP) on Hydrogen peroxide content (µM g-1 fresh weight)of a field pea (*Pisum sativium* L.) genotype under induced cadmium stress at 40 DAS and 60 DAS interval.




Control **T0**:[without $CdCl_2$,SA and SNP];
**T1** [$CdCL_2$ 50 mM];
**T2** [SA 2mM+$CdCl_2$50 mM];
**T3** [SNP100µM+$CdCl_2$ 50 mM];
**T4** [SA 2mM + SNP100µM +$CdCl_2$ 50 mM].

### 4.9 Protein content (mg $g^{-1}$ fresh weight of leaves)

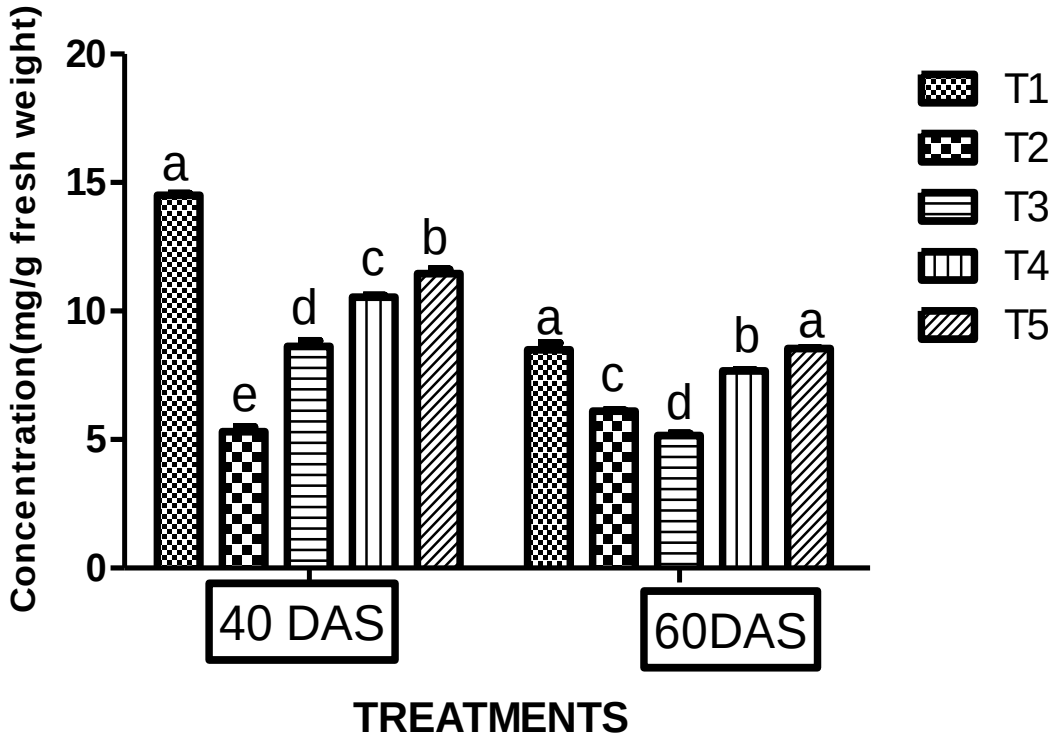
Protein content in the pea plants were reduced under induced cadmium stress. It is observed that maximum protein content 13.31 mg $g^{-1}$ fresh weight of leaves found on 40 DAS in plant whose seeds is untreated (control). The minimum Protein content 6.67 mg $g^{-1}$ fresh weight is found on 40 DAS as compared to other treated plants, respectively in pots( $T_1$) which were non ameliorative and exposed to cadmium stress condition. The value of protein in $T_1$ is nearly two times lesser than the control ($T_0$) observed on 40 DAS .The protein value of $T_2$ is 25% more than $T_1$. The protein value of $T_3$ is 34% more than T1. The best ameliorative effect were shown by $T_4$ (SA+SNP) which showed 42% more protein value as compared to $T_1$ on the basis of 40 DAS.

Table 9 Effect of salicylic acid (SA) and sodium nitroprusside (SNP) on Protein content (mg $g^{-1}$ fresh weight of leaves) of a field pea (*Pisum sativium* L.) genotype under induced cadmium stress at 40 DAS and 60 DAS interval.

| **Treatment** | **Genotype (G) HUDP-15** | | | |
|---|---|---|---|---|
| | **40 DAS** | | **60 DAS** | |
| **$T_0$** | 13.31 | | 8.76 | |
| **$T_1$** | 6.67 | | 6.06 | |
| **$T_2$** | 8.89 | | 5.03 | |
| **$T_3$** | 10.04 | | 7.65 | |
| **$T_4$** | 11.46 | | 8.49 | |
| | **SEm±** | **CD1%** | **SEm±** | **CD1%** |
| | .1451 | 0.481 | 0.130 | 0.414 |

Control **$T_0$**:[without $CdCl_2$,SA and SNP];
**$T_1$** [$CdCL_2$ 50 mM];
**$T_2$** [SA 2mM+$CdCl_2$50 mM];
**$T_3$** [SNP100µM+$CdCl_2$ 50 mM];
**$T_4$** [SA 2mM + SNP100µM +$CdCl_2$ 50 mM].

Figure 8 Effect of salicylic acid (SA) and sodium nitroprusside (SNP) on Protein content (mg $g^{-1}$ fresh weight of leaves) of a field pea (*Pisum sativium* L.) genotype under induced cadmium stress at 40 DAS and 60 DAS interval.




Control **$T_0$**:[without $CdCl_2$,SA and SNP];
**$T_1$** [$CdCL_2$ 50 mM];
**$T_2$** [SA 2mM+$CdCl_2$50 mM];
**$T_3$** [SNP100µM+$CdCl_2$ 50 mM];
**$T_4$** [SA 2mM + SNP100µM +$CdCl_2$ 50 mM].

### 4.10 Malondialdehyde (MDA) content (nM $g^{-1}$fresh weight)

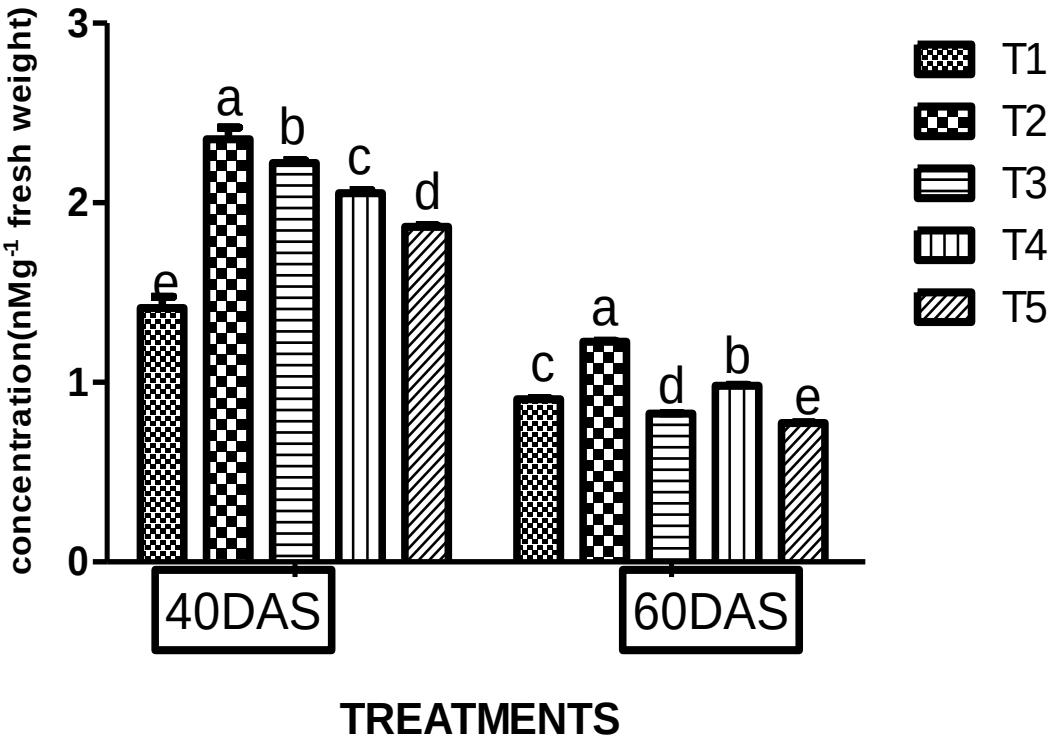
Malondialdehyde (MDA) content in the pea plants were increased under induced cadmium stress. It is observed that minimum MDA (nM $g^{-1}$fresh weight) found in plant leaves whose seeds is untreated (control) and the values were 1.36 nM $g^{-1}$fresh weight on 40 DAS .The maximum MDA content 2.38 nM $g^{-1}$fresh weight is found on 40 DAS as compared to 60 DAS, respectively in pots ( $T_1$) which were non ameliorative and exposed to cadmium stress condition. The value of MDA in $T_1$ is 175% more than the control ($T_0$) observed on 40 DAS .The MDA value of $T_2$ is 5% less than $T_1$. The MDA value of $T_3$ is 12% less than T1. The best ameliorative effect were shown by $T_4$ (SA+SNP) which showed 22% less MDA value as compared to $T_1$ on the basis of 40 DAS.

Table 10 Effect of salicylic acid (SA) and sodium nitroprusside (SNP) on Malondialdehyde (MDA) content (nM $g^{-1}$fresh weight) of a field pea (*Pisum sativium* L.) genotype under induced cadmium stress at 40 DAS and 60 DAS interval.

| **Treatment** | **Genotype (G) HUDP-15** | | | |
|---|---|---|---|---|
| | **40 DAS** | | **60 DAS** | |
| **$T_0$** | 1.36 | | 0.90 | |
| **$T_1$** | 2.38 | | 1.23 | |
| **$T_2$** | 2.25 | | 0.83 | |
| **$T_3$** | 2.08 | | 0.99 | |
| **$T_4$** | 1.86 | | 0.77 | |
| | **SEm±** | **CD1%** | **SEm±** | **CD1%** |
| | 0.042 | 0.135 | 0.013 | 0.004 |

Control **$T_0$**:[without $CdCl_2$,SA and SNP];
**$T_1$** [$CdCL_2$ 50 mM];
**$T_2$** [SA 2mM+$CdCl_2$50 mM];
**$T_3$** [SNP100µM+$CdCl_2$ 50 mM];
**$T_4$** [SA 2mM + SNP100µM +$CdCl_2$ 50 mM].

Figure 9 Effect of salicylic acid (SA) and sodium nitroprusside (SNP) on Malondialdehyde (MDA) content (nM $g^{-1}$fresh weight) of a field pea (*Pisum sativium* L.) genotype under induced cadmium stress at 40 DAS and 60 DAS interval.




Control **$T_0$**:[without $CdCl_2$,SA and SNP];
**$T_1$** [$CdCL_2$ 50 mM];
**$T_2$** [SA 2mM+$CdCl_2$50 mM];
**$T_3$** [SNP100µM+$CdCl_2$ 50 mM];
**$T_4$** [SA 2mM + SNP100µM +$CdCl_2$ 50 mM].

## ANTIOXIDANT ENZYMES ACTIVITY

### 4.11 Ascorbate peroxidise activity (APX) (mg-$^{1}$protein min$^{-1}$)

Ascorbate peroxidises activity (APX) in the pea plants increased under induced cadmium stress. . It is observed that minimum APX (mg-$^{1}$protein min$^{-1}$) found in plants whose seeds is untreated (control) .The maximum 0.95 (mean) mg-$^{1}$protein min$^{-1}$is found on 60 DAS as compared to 40 DAS. The value of APX in $T_1$ is 272.2% more than the control ($T_0$) .The value of $T_2$ is 43% less than $T_1$. The APX value of T3 were 49% less than $T_1$

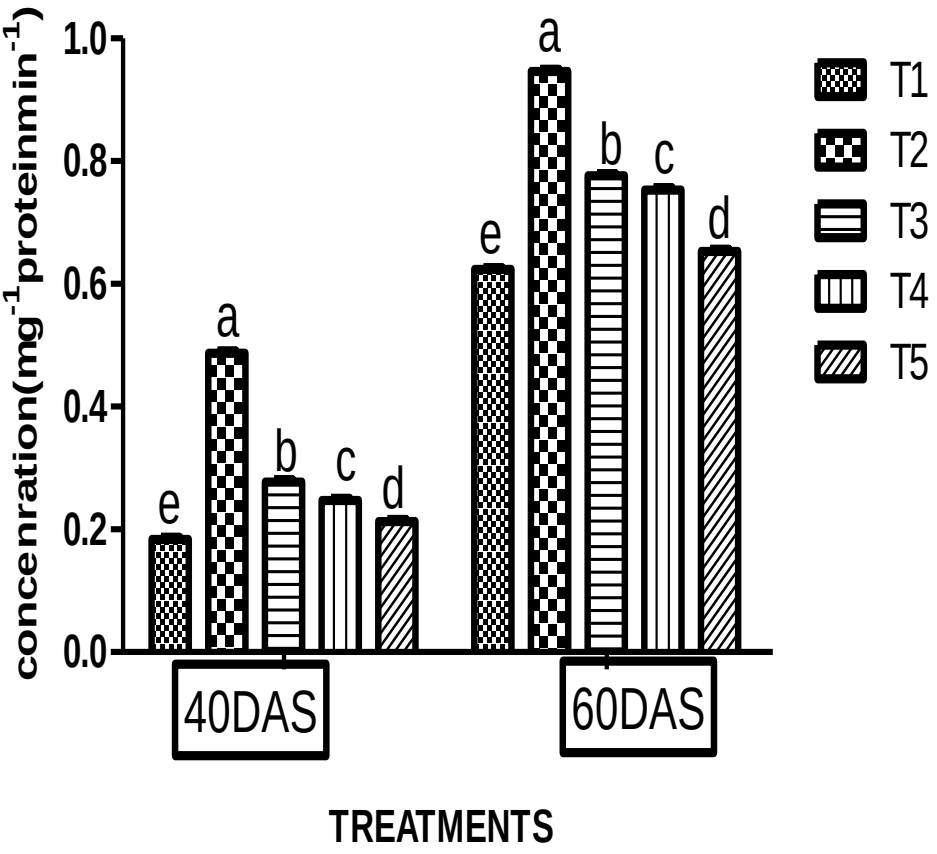
The best ameliorative effect is shown by $T_4$ (SA+SNP) which showed 58% less APX value as compare to $T_1$ on the basis of 40 DAS.

Table 11 Effect of salicylic acid (SA) and sodium nitroprusside (SNP) on Ascorbate peroxidise activity (APX) (mg-$^{1}$protein min$^{-1}$) of a field pea (*Pisum sativium* L.) genotype under induced cadmium stress at 40 DAS and 60 DAS interval.

| **Treatment** | **Genotype (G) HUDP-15** | | | |
|---|---|---|---|---|
| | **40 DAS** | | **60 DAS** | |
| **$T_0$** | 0.18 | | 0.62 | |
| **$T_1$** | 0.49 | | 0.95 | |
| **$T_2$** | 0.28 | | 0.78 | |
| **$T_3$** | 0.25 | | 0.74 | |
| **$T_4$** | 0.21 | | 0.65 | |
| | **SEm±** | **CD1%** | **SEm±** | **CD1%** |
| | 0.05 | 0.15 | 0.01 | 0.12 |

Control **$T_0$**:[without $CdCl_2$,SA and SNP];
**$T_1$** [$CdCL_2$ 50 mM];
**$T_2$** [SA 2mM+$CdCl_2$50 mM];
**$T_3$** [SNP100µM+$CdCl_2$ 50 mM];
**$T_4$** [SA 2mM + SNP100µM +$CdCl_2$ 50 mM].

Figure 10 Effect of salicylic acid (SA) and sodium nitroprusside (SNP) on Ascorbate peroxidise activity (APX) (mg-$^{1}$protein min$^{-1}$) of a field pea (*Pisum sativium* L.) genotype under induced cadmium stress at 40 DAS and 60 DAS interval.




Control **T₀**:[without $CdCl_2$,SA and SNP];
**T₁** [$CdCL_2$ 50 mM];
**T₂** [SA 2mM+$CdCl_2$50 mM];
**T₃** [SNP100µM+$CdCl_2$ 50 mM];
**T₄** [SA 2mM + SNP100µM +$CdCl_2$ 50 mM].

### 4.12 Super oxide dismutase activity (g-1 fresh weight)

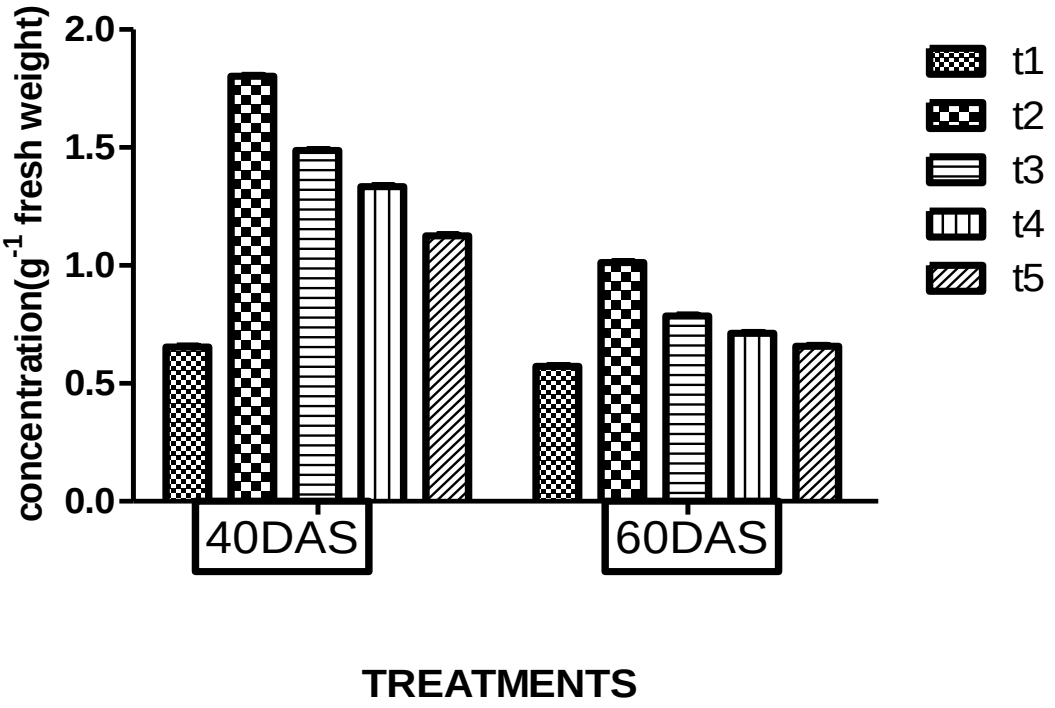
Maximum1.80 (mean) g-1 fresh weight super oxide dismutase concentration is found on 40 DAS as compare to 60 DAS from table 12 and figure 11**.** In this treatment ($T_1$) in stress condition SOD concentration is higher compare to other treatment, it is considered that between the various treatments that the pots ($T_0$) have minimum SOD concentration 0.65 $g^{-1}$ fresh weight on 40 DAS and 0.57 $g^{-1}$ fresh weight on 60 DAS. SA ameliorative and SNP ameliorative plants have higher concentration were observed.SA treated plants have maximum 1.49 $g^{-1}$ fresh weight and SNP treated plants have maximum 1.33 $g^{-1}$ fresh weight.

Table 12 Effect of salicylic acid (SA) and sodium nitroprusside (SNP) on Super oxide dismutase activity (g-1 fresh weight) of a field pea (*Pisum sativium* L.) genotype under induced cadmium stress at 40 DAS and 60 DAS interval.

| **Treatment** | **Genotype (G) HUDP-15** | | | |
|---|---|---|---|---|
| | **40 DAS** | | **60 DAS** | |
| **$T_0$** | 0.65 | | 0.57 | |
| **$T_1$** | 1.80 | | 1.01 | |
| **$T_2$** | 1.49 | | 0.79 | |
| **$T_3$** | 1.33 | | 0.71 | |
| **$T_4$** | 1.13 | | 0.66 | |
| | **SEm±** | **CD1%** | **SEm±** | **CD1%** |
| | 0.06 | 0.14 | .02 | .011 |

Control **$T_0$**:[without $CdCl_2$,SA and SNP];
**$T_1$** [$CdCL_2$ 50 mM];
**$T_2$** [SA 2mM+$CdCl_2$50 mM];
**$T_3$** [SNP100µM+$CdCl_2$ 50 mM];
**$T_4$** [SA 2mM + SNP100µM +$CdCl_2$ 50 mM].

Figure 11 Effect of salicylic acid (SA) and sodium nitroprusside (SNP) on Super oxide dismutase activity (g-1 fresh weight) of a field pea (*Pisum sativium* L.) genotype under induced cadmium stress at 40 DAS and 60 DAS interval.




Control **T0**:[without $CdCl_2$,SA and SNP];
**T1** [$CdCL_2$ 50 mM];
**T2** [SA 2mM+$CdCl_2$50 mM];
**T3** [SNP100µM+$CdCl_2$ 50 mM];
**T4** [SA 2mM + SNP100µM +$CdCl_2$ 50 mM].

# DISCUSSION

The investigation is carried out to access compatibility of salicylic acid (SA) and sodium nitroprusside (SNP) to adverse environmental stress such as salt stress on genotype of pea (HUDP-15). A number of observations were recorded at two stages of the crop growth. *viz.* At 7 days,40 DAS and 60 DAS of plants grown in pots in plant growth chamber into the Department of Physiology, Institute of Agricultural Sciences, Banaras Hindu University. The data obtained were statistically analysed and represented in well organised tables and histograms have been extensively described in the chapter of results. Certain scientific photo plates also have supported the results to a greater extent. The important protein yielding pea treated with SA and SNP grown under induced cadmium stress in soil culture is critically studied and evaluated the compatibility of SA and SNP on recovery of damaged caused due to cadmium stress.

The primary objective of the current study is to evaluate the cadmium-induced modulation in some key morphological, physiological and biochemical attributes of pea. Ghani and Ali (2008) and Ghani (2010) observed depression in growth as well as pod and seed characteristics of pea varieties in response to Cd toxicity. Incipient development of adventitious roots rather than persistence of principle root were observed by Molina *et al*., (2008).

**Morpho-physiological parameters**

Any adverse effect on seed germination is detrimental for the establishment and healthy growth of plants. Cd has been shown to inhibit germination process and the development of seedling (Al- Rumaih *et al*., 2001; Vijayraghavan *et al.*, 2011).The similar observation is as follows that 26% reduction in germination in stress condition as compared to control ($T_0$).As compared to stress ($T_1$), SA ameliorative plant showed 14% more, SNP ameliorative plant showed 20% more and SA+SNP ameliorative plant showed 30% more germination.*


Figure showing general view of an experiment conducted in plant Growth chambers

Inner view of Plant Growth Chamber

Cadmium stress causes a significant reduction on shoots length shoot length's also reduces the synthesis of cell wall components, causes damaged to Golgi apparatus and changes poly saccharides metabolism (Heidai ans Sarani., 2011). Complete inhibition of root elongation occurred in the root tip within the first 12 h of exposure to Cd concentration ranging from 5and 50µM in pine (Schutzendubel *et al*., 2004). In *Brassica juncea* , Cd exhibited inhabiting effect on plant growth, height and biomass accumulation in a dose and time dependent manner( Qadir *et al*.,2004). From our result, it is made known that reduced in plants treated with $CdCl_2$ in the taken pea genotype The similar observation is as follows that 25.4% reduction in shoots length in stress condition as compared to control ($T_0$).As compared to stress ($T_1$), SA ameliorative plant showed 16% more, SNP ameliorative plant showed 26% more and SA+SNP ameliorative plant showed 38% more shoots length.*

Khan et al.(2006) conducted experiments on 5 wheat cultivars at three plants growth stages treated with 0,25,50,and100 mg $kg^{-1}$soil. Nada et al.(2007) exposed almond seedling (*Prunus dulcis* ) to increasing $CdCl_2$ concentration ranging from 0 to 150 µM cdcl2 and observed that root growth is affected significantly only by 150 µM $CdCl_2$ they further noticed that Cd particularly reduced leaf growth in terms of dry weight , but even more sensitively leaf surface area. According to Farooq *et al*., (2009) NO regulates strategies responsible for salinity resistance. When this signalling molecule reaches a plant before initiation of stress, it triggers reactions which lead to increase in leaves antioxidants bustle and higher potential for K assimilation under salinity stress, as a result the plant become more salinity tolerant before $CdCl_2$ comes to play.. Pre-treatment or at least, NO application at early phases of stress seems a better strategy for protection because plants may avoid the stress effects or tolerate it better (Wahid and Ghani, 2008). The similar observation is as follows that 23% reduction in number of leaf $plant^{-1}$ in stress condition as compared to control ($T_0$).As compared to stress($T_1$), SA ameliorative plant showed 11% more , SNP ameliorative plant showed 20% more and SA+SNP ameliorative plant showed 47% more number of leaf $plant^{-1*}$.

For leaf area 22% reduction in number of leaf area $plant^{-1}$ in stress condition as compared to control ($T_0$).As compared to stress($T_1$), SA ameliorative plant showed

9% more , SNP ameliorative plant showed 32% more and SA+SNP ameliorative plant showed 27% more number of leaf area plant$^{-1*}$

Besides of the antioxidative effect of NO (Beligni *et al*., 2002), this compound can lead to reduction in Na/K ratio in shoots and roots (our study, data not shown) what additionally increases plants tolerance for saline environment.

Moreover, proteomic analyses showed that two superoxide dismutase were promoted by SA in *Arabidopsis* germinating seeds, which might contribute to an enhanced antioxidant capacity (Rajjou *et al.*, 2006). SA treatment (0.5 mM for 24 h) also causes a strong up-regulation of translation initiation and elongation factors, proteases, and two subunits of the 20S proteasome, partisan the hypothesis that SA improves seed germination by promoting the synthesis of proteins that were essential for germination, and the enlistment or degradation of seed proteins accumulated during seed maturation. Germination is not affected by high salinity (Borsani *et al.*, 2001). This appwerent discrepancy is due to the antioxidant effect of catechol, the product of the salicylate hydroxylase that accumulates in the *NahG* seeds and seedlings (Lee *et al.*, 2010). External application of SA also partially reverses the inhibitory effect of oxidative (0.5 mM paraquat) and heat stress (50 °C for 3 h) on seed germination (Alonso-Ramírez *et al.*, 2009). In addition, the biosynthesis of several enzymes involved in metabolic pathways such as the glyoxylate cycle, the pentose phosphate pathway, glycolysis, and gluconeogenesis is also strongly activated by SA, suggesting that SA promotes the release from a quiescence state to the establishment of a vigorous seedling (Rajjou *et al.*, 2006)

**Biochemical parameters and antioxidant enzyme activity**

The present results focus upon two major studies biochemical parameters: chlorophyll content, protein, hydrogen peroxide, antioxidant enzyme activity and the reactive oxygen species, which is affected due to cadmium stress on the one hand as well as its conspicuous alleviation to certain extent by the plant growth regulator salicylic acid and sodium nitroprusside.

The reduction of biomass by Cd toxicity could be the direct consequence of the inhibition of chlorophyll synthesis (Padmaja *et al*., 1990) and photosynthesis (Bazzaz *et al*., 1975. Baszynski *et al*., 1980). Some studies reported a marked in lowering the photosynthetic rate for different plant species under exposure to Cd stress (Sawhney *et al*., 1990. Sheoran *et al*., 1990a. 1990b). when growing the vegetables plants in medium with high level of Cd showed negative effect in photosynthetic processes, such as chlorophyll content and photosynthesis (Baszynski *et al*., 1980. Padmaja *et al*., 1990. Satyakala, 1997), the activities of related enzymes (Ouariti *et al*., 1997 a., 1997b. Ascencio and Cedeno Maldonado, 1979) and photochemical reaction (Li and Miles, 1975. Skorzynska and Baszynski, 1995)..In the present investigation, almost similar observations were recorded (Table 5, 6, 7 and Fig. 4, 5, 6). When extent of damaged in term of percentage as comparedd to control is calculated, it is observed that percent reduction in these parameters in pea .In pea 50µM $CdCl_2$ cause changing chlorophyll a chlorophyll b, total chlorophyll contents as 27% reduction in chlorophyll a 15% reduction in chlorophyll b and 26% reduction in total chlorophyll contents. The effects were reduced by ameliorates as 15% more chlorophyll a ,17% more chlorophyll b and 48% more total chlorophyll contents by comparing to the stress value $(T_0)^*$

The decrease in protein under the stress of cadmium and lead in *Vigna* and Hydrilla plants is reported by Bhattacharya and Choudhari (1994), such decrease in protein content under the heavy metal stress is also reported by Vyas and Puranik (1993). Chaoui *et al.* (1997) reported decrease in protein content in *Phaseolus vulgaris* roots by the treatment of cadmium and zinc. There is significant decrease in protein content in the leaves of *Artemisia annua* (Khudsar *et al*., 2004). Almost similar observations were recorded (Table 9 and Fig. 10). When extent of damaged in term of percentage as comparedd to control is calculated, it is observed that percent reduction in these parameters in pea. In pea 50µM $CdCl_2$ cause changing protein contents as 50% reduction in protein content. The effects were reduced by ameliorates as 33% more protein contents in SA treated ,50% more protein contents in SNP treated and72 % more protein contents in SA+SNP treated by comparing to the stress value $(T_0)^*$

Spots of $H_2O_2$ and $O^{-2}$ in leaves of rapeseed and the contents of $H_2O_2$ and MDA have been increased considerably in Cd affected rapeseed plants clearly indicating oxidative damaged corroborating the results of previous studies (Dong *et al.*, 2006; Hu *et al.,* 2009; Hasanuzzaman *et al*., 2012a). $H_2O_2$ pre-treatment reduced oxidative damaged by decreasing the spots of $O^{-2}$ and $H_2O_2$ and reducing the amount of $H_2O_2$ and MDA contents against Cd toxicity (Hu et al., 2009), reducing contents of MDA and $O^{-2}$ in salinity affected wheat plants (Li *et al*., 2011), decreasing $O^{-2}$, $H_2O_2$and MDA in chill affected cucumber seedlings (Zhang *et al*., 2011). The results of these previous reports indicate the decisive functions of $H_2O_2$ in reducing oxidative stress. At low concentration, $H_2O_2$ can as signalling molecule which modulates various genes related to stress defence mechanism. $H_2O_2$ implicated NO-mediated ABA-induced activation of mitogen-activated protein (MAP) kinase cascade which modulated antioxidant defence mechanism maize leaves. $H_2O_2$ can modulate NO and NO itself is an ROS scavenger (Zhang *et al.,* 2007). In the present investigation, almost similar observations were recorded (Table 8 and Fig.7). When extent of damaged in term of percentage as comparedd to control is calculated, it is observed that percent reduction in these parameters in pea .In pea 50µM $CdCl_2$ cause increasing $H_2O_2$ content 3.7 times the control ($T_1$) one. The $H_2O_2$ content were reduced by ameliorates SA as 40%, $H_2O_2$ content were reduced by ameliorates SNP as 40%, $H_2O_2$ content were reduced by ameliorates SA+SNP as 71%, by comparing to the stress value $(T_0)^*$

From Table10 and Fig.9 it is observed that when extent MDA in term of percentage as comparedd to control is calculated, it is observed that percent reduction in these parameters in pea .In pea 50µM $CdCl_2$ cause increasing MDA content 50% more as compared to the control ($T_1$) one. The MDA content were reduced by ameliorates SA as 10%, MDA content were reduced by ameliorates SNP as 12%, MDA content were reduced by ameliorates SA+SNP as 14%, by comparing to the stress value $(T_0)^*$.

Various authors working on different plant species that an activity of SOD, APX, GR, CAT and POD increases under salinity stress (Ahmad et al. 2010; Koyro et al. 2012). Exogenous application of NO increased activity of CAT, SOD, POD and

APX in seashore mallow (Guo et al. 2009), mustard (Zeng et al. 2011), wheat (Ruan et al. 2002), chickpea (Sheokand et al. 2010), and protected plants from oxidative damaged under salt stress. , SA could contribute to maintaining cellular redox homeostasis through the regulation of antioxidant enzymes activity (Durner and Klessig, 1995, 1996; Slaymaker *et al.*, 2002) and induction of the alternative respiratory pathway (Moore *et al.*, 2002), and to regulating gene expression by inducing an RNA-dependent RNA polymerase that is important for post-transcriptional gene silencing (Xie *et al.*, 2001). Observations were recorded (Table11, 12 and Fig 10, 11. ). it is is found that when extent APX and SOD activity in term of percentage as comparedd to control is calculated, it is observed that percent reduction in these parameters in pea .In pea 50µM $CdCl_2$ cause increasing APX and SOD content 170% and300% more as compared to the control ($T_1$) one. The APX and SOD content were reduced by ameliorates SA as 40% and17%, APX and SOD content were reduced by ameliorates SNP as 50% and26%, APX and SOD content were reduced by ameliorates SA+SNP as 58% and37%, by comparing to the stress value $(T_0)^*$.

The present work leaves enough scope for manipulations of the application of SA and SNP for different crop verities under different afro climatic conditions, especially in overcoming cadmium stress apart from extending various academic work of vital significance. With such practices, production one of the grain legume pea (*Pisum sativum L.*) under stress may be maintained to a certain extent, which hopefully would be also effective for other pulses.

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*Chapter V*

# SUMMARY AND CONCLUSION

The **field pea** *Pisum sativum*(2n=14) is a type of pea , often called *P. sativum* sub sp. *arvense* (L.) Field peas were one of the old most adopted domesticated crops, cultivated for at least 7,000 years. They were now grown in many countries for both human consumption and stock feed. It is a climbing annual legume with weak, vinyl, and partially succulent stems. Vines often were 4 to 5 feet (120 to 150 cm) long, but when grown alone, field pea's weak stems prevent it from growing more than 1.5 to 2 feet (45 to 60 cm) tall. Leaves have two leaflets and a tendril. Flowers were white, pink and purple. The seeds may be planted as soon as the soil temperature reaches 10 °C (50 °F), with the plants growing best at temperatures of 13 to 18 °C. They do not flourished in the summer heat of warmer temperate and lowland tropical climates.

Present research work carried out in order to estimate the Cd stress effect on growth parameters Cadmium (Cd) is a highly toxic in nature among heavy metal which causes oxidative stress in plants and has a high level of toxicity for plants, animals and human in bean seedling. Cd in soil causes many disturbances in mineral nutrition and carbohydrate metabolism; reduce biomass, inhibition of chlorophyll synthesis, deleterious effect in photosynthetic processes. Nitric oxide is involved in germination and initiation of lateral roots.

Salicylic acid (SA) is one of the endogenous an important PGR which regulators, naturally occurring signalling molecule and its major role in growth and development of plants. Salicylic acid is important compound required in many physiological processes such as photosynthesis, nutrient uptake and transport, flowering and inhibition of fruit ripening.

When plants were pre-treated with SNP it triggers reactions which lead to increase in leaves antioxidants bustle and higher potential for K assimilation under salinity stress, as a result the plant become more salinity tolerant before $CdCl_2$ comes

to play., they become pre-conditioned to better tolerance to the salt stress. This is one of the important factors of the higher yield, shoot and root fresh and dry weight. Strong evidences indicate that NO acts as a second messenger, triggering different cellular responses, such as the increase in antioxidant defence systems

Literature consulted in this regard has been extensively presented in the section of review of literature. Hence an experiment is conducted to evaluate effect of salicylic acid (SA) and sodium nitroprusside(SNP) on morpho-physiological, biochemical and antioxidant enzyme activity of pea under induced cadmium toxicity.

The present investigation is carried out in soil culture by inducing cadmium toxicity with $CdCL_2$ @ 50 mM in Plant growth chamber in the Department of Plant Physiology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi. There were five treatments including control as follows:

Control **$T_0$**:[without $CdCl_2$,SA and SNP];**$T_1$** [$CdCL_2$ 50 mM]; **$T_2$** [SA 2mM+$CdCl_2$50 mM];**$T_3$** [SNP100µM+$CdCl_2$ 50 mM]; **$T_4$** [SA 2mM + SNP100µM +$CdCl_2$ 50 mM].

The experiment is conducted in factorial randomized block design with five individual treatments and each treatment is replicated three times. Observations were made on certain morpho-physiological, biochemical, antioxidant enzymes activity, *viz*. germination percentage, length of shoot, l, number of leaves per plant, leaf area, chlorophyll b, total chlorophyll, , protein content, hydrogen peroxide, Ascorbate peroxidise, malondialdehyde content and superoxide dismutase activity.

The observation for germination percentage is recorded at 7 DAS in growth chamber also the observations on morpho-physiological parameters were taken at 40, and 60 DAS in the Plant growth chamber.

From this present study, it can be concluded that the application of $CdCl_2$ adversely affected the growth and defence mechanism as well as metabolism of pea plants. Heavy metal tolerance is a complex phenomenon in plants, and various research methodologies and genetic approaches were used to characterize the diverse biochemical events that occur in response to cadmium stress. High concentrations of

cadmium induce stunted growth and loss in chlorophyll content as well as oxidative damageds by altering antioxidant machinery, leading to membrane damaged through lipid peroxidation. The application of phytohormones such as SA and SNP as in presence of $CdCl_2$ in height concentrations play an antagonistic role in cadmium uptake. Thus, their use in cadmium contaminated soil may help to grow pea plants with normal growth.

Germination and seedling establishment were sensitive stages of plants growth to cadmium stress. Cadmium toxicity mainly causes delay in germination and reduction in germination rate. So, the success of plant species under cadmium toxic conditions is that first, they should be able to maintain their lives and then, when the cadmium level is decreased, germination is began. Cadmium toxicity affects the seeds germination and growth by reducing the water potential and toxicity of specific ions and reducing nutrition requirements vegetative traits such as radical and shoot length, dry weight of radical and plumule, and thus, total dry weight is reduced in cadmium stress. Salicylic acid (SA) 2mM and Sodium nitroprusside (SNP) 100µM priming increased the number of leaves in the plants treated with $CdCl_2$. Significant reduction in total leaf area is observed with respect to $CdCl_2$, as comparedd to control.

In present experiment the combined application of SA 2mM and SNP 100µM along with $CdCl_2$ in pea plants altered the activities of antioxidant enzymes in comparison to $CdCl_2$ treated alone. it is is found that APX and SOD activity in term of percentage as comparedd to control is found, it is observed that percent reduction in these parameters in pea .In pea 50µM $CdCl_2$ cause increasing APX and SOD content 170% and300% more as compared to the control ($T_1$) one. The APX and SOD content were reduced by ameliorates SA as 40% and 17%, APX and SOD content were reduced by ameliorates SNP as 50% and 26%, APX and SOD content were reduced by ameliorates SA+SNP as 58% and 37%, by comparing to the stress value $(T_0)^*$.

Last but not the least keeping in view the facts regarding. This may be concluded that Salicylic acid (SA) 2mM and Sodium nitroprusside (SNP) 100µM has positive effects on morpho-physiological, biochemical and antioxidants enzymes

activity under induced cadmium stress in pea. Therefore, Salicylic acid (SA) and Sodium nitroprusside (SNP) could be utilized effectively for combating cadmium stress.

*@40 DAS

**FUTURE PROSPECTUS**

Studies conducted on Salicylic acid (SA) and Sodium nitroprusside (SNP) over a period of years have established several vital functions they perform in the growth and development of plants. Salicylic acid (SA) and Sodium nitroprusside (SNP) research is paving along different lines of enquiry:

1. Their effect characterization from different plants and plant parts.
2. To delineate different biosynthetic pathways operating in plants.
3. Detailed examination of participation of Salicylic acid (SA) and Sodium nitroprusside (SNP) in the development of photosynthetically active chloroplasts.
4. Analysis of the role of Salicylic acid (SA) and Sodium nitroprusside (SNP) in photosynthetic electron transfer.
5. Role of Salicylic acid (SA) and Sodium nitroprusside (SNP) in growth and development of plants with the help of SA and SNP insensitive mutants.
6. Deciphering molecular mechanisms of SA and SNP action by cloning of SA and SNP regulated genes.
7. Their mode of action at the membrane and gene levels.
8. Interactions between SA, SNP and conventional plant hormones.
9. Development of technology for the improvement of agricultural production by the use of SA and SNP.

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