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EFFECT OF CADMIUM ON RESPIRATION AND ENZYME ACTIVITY IN GERMINATING PEA (*PISUM SATIVUM*) SEEDS

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ABSTRACT: The present investigation were conducted to ascertain the effect of cadmium on pea (*Pisum sativum* L.cv.Azad P-1) germinating seeds on respiration and certain enzymes (amylase, protease, phosphatase, peroxidase).Activity of enzymes was assayed 2,4,6 and 8 days after germination in embryonic axis as well as cotyledons. In control seeds, the rate of respiration showed a biphasic increase. Higher concentration of Cd^{2+}suppressed the rapid rise in respiration during second phase (3 days after germination) and this phase was, completely eliminated in presence of 2mM and 3mM Cd^{2+}. Total amylase activity in embryonic axis decreased in presence of cadmium, decrease was proportional to cadmium concentration. The influence of cadmium on protease followed a pattern similar to that of total amylase in both embryonic axis as well cotyledons, however, the effect on protease was greater than that on total amylase during initial stage. Thus at 1 mM Cd^{2+}, the activity of protease was 60% of control on the day 6 after germination. The corresponding values for 3 mM Cd^{2+} for same day was 57% of control. Activity of acid phosphatase was also significantly depressed by cadmium. On the 8^{th} day1, 2 and 3 mM Cd^{2+}, 44, 72 and 80% decrease in enzyme activity in embryonic axis and 21, 66 and 75% decrease in cotyledons for the respective treatments respectively. The decrease in activity of the enzymes was not linear. Peroxidase activity showed inverse relation with cadmium treatment, peroxidase activity was higher in cadmium treated seeds as compared to distilled water control, and the activity of the enzyme was dependent on the concentration of cadmium in the medium.

KEYWORDS: Cadmium, respiration, amylase, protease, phosphatase, Peroxidase.

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1. INTRODUCTION

For the last few decades, there has been an increasing concern over the unabated and rapid pollution of the environment, largely owing to the relentless exploitation of the surroundings by the mankind. The release of toxic waste in the environment is increasing day by day [1-3]. The uncontrolled disposal of the industrial effluent and the solid wastes has become a matter of great concern as these are known to contain heavy metals such as Cd^{2+}, Pb^{2+}, Ni^{2+} etc [4-6]. They are also added to soils through the application of some phosphorus fertilizers [7]. Plants is a member of food chain and creates a risk for man and animals through contamination of food supplies. The food contaminated with heavy metals causes various diseases in human being and animals [8]. Besides endangering human health the heavy metals are likely to jeopardize the welfare of mankind by their impact on productivity [9-12]. The need of food for the growing population is not met since more and more of the cultivable land is being converted to non cultivable due to severe heavy metal contamination. Cadmium when taken up cause erythrocyte destruction, renal degradation, hypertension, various heart diseases, bronchitis, cancer, gastroenteritis, osteoporosis and the itai-itai diseases [13]. Although the heavy metals are not essential for plant growth they are readily taken up directly from air or indirectly through the contaminated soil and water by the plant species and get accumulated in various parts. It is reported that heavy metals are phytotoxic [14]. The amount of heavy metals absorbed by the plants tend to increase with increase in concentration of the heavy metals in the soil [15-16]. Respiratory metabolism supplies energy, reductants as well as intermediates for various biosynthesis reactions and is of pivotal importance for growth of embryo axis[17]. Seed germination is the primary stage in the establishment of a plant and is known to be sensitive to cadmium. Use of reserve nutrients of seeds during germination plays a key role in growth of the seed. The present work is designed to analyse the effect of cadmium on respiratory activity, activities of enzymes in germinating seeds of *Pisum sativum.*

2. MATERIALS AND METHODS

Certified pea seeds (*Pisum sativum* L.cv.Azad P-1)were purchased from a local seed dealer. Seeds were surface sterilized with 1% sodium hypochloride solution for 5 minutes and thoroughly washed a few times with sterile water. Placed two layer of moistened Whatman No.1 filter paper discs in petri dishes. Added 10 ml of either sterile distilled water (control) or solution of cadmium acetate in petri dishand added 10 seeds of uniform weight in each of them. Seeds were germinated at 25^{o}C in the dark, in an incubator. Seeds were sampled 2, 4, 6 and 8 days after germination, for analysis of parameters. Rate of respiration of germinating seeds was determined from the rate of oxygen consumption using Clark electrode. Decoated seeds after slicing each cotyledon into two pieces were placed in a sealed reaction vessel which contained 50 ml of 0.05 M phosphate buffer (pH 7.5). The reaction vessel was placed in a circulating water bath. Temperature was maintained at 30^{0}C.

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The rate of oxygen depletion from the media was recorded over a period of 10 min. For preparation of cell free extract, the germinating seeds were removed, thoroughly washed under running tap water and then twice with distilled water. Two seeds was taken and cotyledons and growing embryonic axis were homogenized separately in 5 ml. of grinding mixture consisting of 50 mM Tris containing 5 mM cysteine hydrochloride adjusted to pH 7.5 in chilled pestle and mortar. Thehomogenized tissue was centrifuged at 15,000 RCF for 30 min at 4^0C (Remi model 30CPlus) the supernatant obtained was dialysed against x4 diluted extraction buffer for two hours at 4ºC with stirring.

2.1Enzyme Assays

Amylase was assayed by the method of Swain and Deeker (1966) [18]. Substrate 1% soluble starch in 0.1 M acetate buffer (pH 5.6).To 0.5 ml of extract added 1 ml of the substrate. After 10 min. of incubation at 25ºC, 2 ml of 3, 5 dinitrosalicyclic acid reagents was added and the tubes placed in a boiling water bath for 5 min., added 10 ml water after cooling. The absorbance recorded at 540 nm. Calibration curve was made using Maltose.For acid phosphatase assay the method of Johnson and Holloway (1973) [19] was used. To each fraction was added 0.5 mg p-nitrophenyl phosphatase dissolved in 1.0 ml, 0.2 M acetate buffer of pH 5.5. The reaction mixture was incubated for 10 min. Reaction was stopped with 2 ml of 10% sodium carbonate solution at the absorbance measured at 410nm. Calibration curve was made with p-nitrophenol. For protease assay the method of Beevers (1968) was used. 1 ml of extract was incubated with 1 ml of 1 % casein and 1 ml of 0.2M phosphate buffer pH 7.0. The mixture was incubated at 40ºC for 90 min. The reaction was terminated by the addition of 1 ml of 20% TCA. The precipitates were removed by centrifugation at 3000 RCF for 15 min, the clear supernatant was used for the determination of amino acid at 570 nm. For peroxidase the method described by Seevers (1971) [20] was used. Total peroxidase in extract were carried out at 25ºC using benzidine as substrate. The benzidine reaction mixture consists of 1.9 ml of 20 mM sodium acetate pH 5.0, 0.4 ml of 1.3mM benzidine in 0.2 M sodium acetate pH 5.0, 0.1 ml 1% aqueous ammonium molybdate, 0.1 ml of 30 mM $H_2O_2$and 0.1 ml of enzyme solution. Absorbance was recorded at 334 nm.

3. RESULTS AND DISCUSSION

Effect of Cd^{2+} on rate of respiration of germinating pea seeds is presented in Table 1. In control seeds, the rate of respiration showed a biphasic increase. A similar biphasic increase in respiratory activity also occurred in presence of low concentration of Cd^{2+} (1mM) except that the rate was lower and the steady phase (between days 2 to 3) was extended to 4 days and the respiratory rate began to decline on 6^{th} day. Higher concentration of Cd^{2+}suppressed the rapid rise in respiration during second phase (3 days after germination) and this phase was, in fact completely eliminated in presence of 2mM and 3mM Cd^{2+}. On the 6^{th} day, seeds germinated in 1mM, 2mM and 3mM Cd^{2+} was 50.3%, 35.9% and 27.9% of the control respectively. Effect of Cd^{2+} on total amylase activity,

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in embryonic axis and cotyledons in presented in Table-2. In control seeds, the level of amylase activity in embryonic axis increased almost linearly upto 8 days after germination. During the first 2 days in the case of embryonic axis, total amylase activity was comparable to that in control at the lowest concentration of Cd^{2+}. However, thereafter there was an appreciable decrease in activity of this enzyme. The depression in activity was proportional to the concentration of Cd^{2+}. Eight days after germination, the level of this activity in germinated seeds in 1,2 and 3mM Cd^{2+} was 60, 43 and 21% that of controls. Results also indicate that during germination, the extent of inhibition increased with time at all the three concentration of Cd. In presence of 1mM Cd^{2+} the total amylolytic activity was depressed by 93, 88, 73 and 60% on 2, 4, 6 and 8 days of germination respectively. The corresponding values for 2mM Cd^{2+} and 3mM Cd^{2+} were 87%, 75%, 43% and 78%, 63%, 32%, 21% respectively for 2, 4,6 and 8 days after germination. In case of cotyledons in control as well as Cd treated seeds showed increase in activity of amylolytic, followed by an decrease in activity. Other trends were similar to those of embryotic axis. This activity of total amylase at 1, 2 and 3 mM Cd^{2+} on days 2, 4, 6 and 8 days after germination were 79%, 71%,66% and 51%. 73%, 57%, 39%and 27%. 70%, 49%, 27%, and 23% respectively. The protease activity is presented in Table-3. In control seeds, protease activity increased rapidly between days 4 and 6 and declined rapidly on day 8 in case of embryonic axis. However, in case of cotyledons this decline on day 8 was not as rapid. The influence of cadmium on protease followed a pattern similar to that of total amylase in both embryonic axis as well cotyledons, however, the effect on protease was greater than that on total amylase during initial stage. At later stage the influence of cadmium on protease was less pronounced then on total amylase activity. Thus at 1 mM Cd^{2+},the activity of protease was 58, 71, 60 and 72% of control on the day 2,4,6 and 8 after germination. The corresponding values for 3 mM Cd^{2+} for these days were 69, 54, 57 and 56% of control respectively.Table-4 represents the activity of acid phosphatas. The activity of acid phosphatase in control seeds in embryonic axis as well as cotyledons showed a steady increase with time. Activity of acid phosphatase was significantly depressed by cadmium. The effect of Cd^{2}on embryonic axis and cotyledons was similar. On the 8^{th} day1, 2 and 3 mM Cd^{2+} 44, 72 and 80% decrease in enzyme activity in embryonic axis and 21, 66 and 75% decrease in cotyledons respectively. Results also indicate that during germination, the extent of inhibition increased with time in seeds germinated at all the three concentration of Cd.The total activity of peroxidase is presented in Table-5. A substantial activity of peroxidase was detected in seeds germinated in water on 2^{nd} day of germination which increased linearly in embryonic axis as well as cotyledons upto 4 days of germination and then begins to decline Peroxidase activity showed inverse relation with cadmium treatment, peroxidase activity was higher in cadmium treated seeds as compared to distilled water control, and the activity of the enzyme was dependent on the concentration of cadmium in the medium. The general trend

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observe was that distilled water treated seedlings had minimum activity while higher concentration of heavy metal treated seedlings had maximum activity. The peroxidase activity increased with concentration of cadmium.

Discussion

The data in Table 3 show that respiratory activity , as measured by the rate of oxygen uptake , in control seeds remained at a low constant level during the first 24hrs, increased by about 50% in next 24hrs and after remaining steady up to 72hrs, increased almost linearly up to 6^{th} day. Such uneven and phasic changes in respiratory activity of germinating seeds, including that of pea, have been observed earlier as well [21-23]. Kolloffel as described in the initial increase in respiration of germinating pea seeds to hydration of tissues which results in activation of the respiratory enzyme already present in mature seeds. A similar view was also advanced by Opik (1965) [24] while investigating the changes in respiratory activity of germinating mung bean seeds. During these phase maturation of mitochondria is also initiated. This process involve development of the vesicular mitochondria with a few cristae to critae richoues [25-26], transport of cytoplasmic proteins and phospholipids into mitochondria [27] and all these transformations are accompanied by an increase of biological activityof mitochondria.[27-28]. The precise cause for the third phase,during which a linear enhancement in respiration occurs, have not been established unequivocally but may be associated with either improved respiratory efficiency of matured mitochondria and biogenesis of additional mitochondria [29]. The second phase of study respiration rate represents a situation where hydration dependent activation of enzymes is fully achieved but mitochondria continue to undergo maturation [29].In the seed of soyabean [30] and Avena fatua [31] the respiration during first few hours of germination has been found to be pre-dominantly cyanide resistant. In control the respiratory activity in seeds of germinating chick pea [32], black gram [33] and peas [34] has been characterized by its extreme toxicity to cyanide. During the present investigations, cadmium was found to suppress the increase in respiratory activity of all the phases but it most pronounced deleterious effects was on the third phase (after 3 days of germination) during which a rapid and linear increase in respiration occurred in seeds kept for germination in water. Infact, this phase was almost completely abolished in presence of 1 mM Cd^{2+} . Hence Cd^{2+} seems to interfere with respiratory activity by affecting the activation of enzymes, mitochondria maturation and perhaps biogenesis of mitochondria as well. Activity of totalamylase was found to increase after about 4 days in cotyledons of germinating seeds[35-39].As in clear from the results in Table-2 Cadmium depresses activities of amylase in germinating seeds and thismay hamper the supply of glucose for respiratory process. During germination, mobilization of cotyledons reserve proteins in mediated by protease[40-42]. In control protease activity increased rapidly between 2 and 6 days . Increase in casseolytic activity during initial stages of germination has been reported by [40 and 43]. Cadmium

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decrease protease activity and similar reduction has been reported by [43] in case of chromium. Acid phosphatase is an important enzyme of phosphate metabolism and is considered to play important role during seed germination [44] as shown in Table-4, acid phosphatase activity was significantly depressed by cadmium. These results are in conformity with those of [43; 45 and 46]. Peroxidase induction is a general response of higher plants to exposure to heavy metals as well as other stress including injury. It has been observed in various tissues in many species of plants in response to various heavy metals [47-50].

4. CONCLUSION

It is concluded that cadmium disturbs the metabolism of carbohydrates, phosphate and proteins. Results of this study support the postulate that cadmium through its profound effect on activities of hydrolytic enzymes such as amylase is likely to interfere with mobilization of starch. The metabolism of compounds containing phosphorus would be further disturbed due to the deleterious effect of cadmium on acid phosphatase. it is also evident that the respiratory activity of germinating seedswould be impaired due to a restricted supply of glucose because of depressed activities of amylase .all of these effects may partly account for the inhibition of seed germination by cadmium. The release of amino acids from hydrolysis of seed storage proteins may however be another cause, because protease activity was also decreased by cadmium.

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Table-1. Rate of respiration of pea seeds germination subjected to cadmium (µ mol O_2consumption $Seed^{-1}$ $hour^{-1}$).

Treatment	Days after germination							
	1	2	3	4	5	6	7	8
Control	1.52 ± 0.21 (100)	2.84 ±0.26 (100)	2.72 ± 0.30 (100)	3.52 ±0.38 (100)	3.80 ±0.41 (100)	4.01 ±0.48 (100)	4.12±0.47(100)	4.04±0.45 (100)
1 mM Cd	1.40 ± 0.18 (92.1)	2.16±0.22 (87)	2.28 ± 0.21 (83.8)	2.20 ±0.26 (62.5)	2.24 ±0.28 (58.9)	2.02 ±0.22 (50.3)	1.92±0.24(46.6)	1.76±0.25 (43.5)
2 mM cd	1.12±0.13 (73.6)	1.68 ±0.19 (67.7)	1.84 ± 0.20 (67.6)	1.58 ±0.23 (44.8)	1.46 ±0.18 (38.4)	1.44 ±0.16 (35.9)	1.20±0.18(29.1)	1.02±0.09 (25.2)
3mM cd	1.04 ± 0.08 (68.4)	1.48 ±0.17 (59.6)	1.55 ± 0.18 (56.9)	1.32 ±0.19 (37.5)	1.20 ±0.11 (31.5)	1.12 ±0.13 (27.9)	0.96±0.07(23.3)	0.88±0.06 (21.7)

Mean of three replicates ± S.D; Values in parenthesis denotes enzyme activity as % of control.

Table-2. Total amylase activity [µ moles (maltose liberated) $seed^{-1}min^{-1}$] in the embryonic axis and cotyledons of the pea seeds germination in the presence of cadmium.

Treatment	Embryonic Axis				Cotyledons			
	2nd Day	4th Day	6th Day	8th Day	2nd Day	4th Day	6th Day	8th day
Control	12.87±0.21 (100)	21.52±0.15 (100)	63.50±0.42 (100)	69.85±0.25 (100)	8.04±0.19 (100)	12.54±0.25 (100)	28.82±0.32 (100)	25.86±0.24 (100)
1 mM	11.96 ±0.24 (93)	18.92±0.36 4(88)	6.37±0.42(73)	42.06±0.31 (60)	6.3 ±0.74 (79)	8.91 ±0.65 (71)	18.98±0.74 (66)	13.26±0.23(51)
2 mM	11.21±0.56(87)	16.16±0.68 (75)	38.11±0.35 (60)	30.08±0.34 (43)	5.88±0.35(73)	7.12 ±0.65 (57)	11.18±0.35 (39)	6.93±0.14(27)
3 mM	9.98±0.56 c(78)	13.66±0.65 (63)	20.13±0.32 (32)	14.62±0.74 (21)	5.66±0.65 (70)	6.18 ±0.09 (49)	7.91±0.39 (27)	6.03 ±0.35 (23)

Mean of three replicates ± S.D; Values in parenthesis denotes enzyme activity as % of control.

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. **Table-3.** Protease activity [µ moles (amino acids released) seed^{-1} min^{-1}] in embryonic axis and cotyledons of pea seed in presence of cadmium

Treatment	Embryonic Axis				Cotyledons			
	2nd Day	4th Day	6th Day	8th Day	2nd Day	4th Day	6th Day	8th day
Control	2.69±0.32 (100)	7.28±0.65 (100)	17.26±0.58 (100)	11.04±0.6 (100)	1.32±0.85 (100)	3.27±0.32 (100)	8.16±0.52 (100)	7.96±0.51 (100)
1 mM	2.20±0.25 (82)	5.17±0.81 (71)	14.7±0.71 (85)	9.45±0.58 (86)	1.01±0.68 (77)	2.02±0.85 (62)	4.98±0.58 (61)	4.01±0.61 (50)
2 mM	1.70 ±0.58 (63)	5.66±0.42 (78)	11.34±0.25 (65)	8.63±0.85 (78)	0.89±0.42 (67)	1.89±0.17 (88)	4.78±0.85 (59)	4.57±0.26 (57)
3 mM	1.56±0.41 (58)	5.19±0.23 (71)	10.4±0.51 (60)	7.91±0.35 (72)	0.91±0.25 (69)	1.78±0.58 (54)	4.62±0.62 (57)	4.45±0.39 (56)

Mean of three replicates ± S.D; Values in parenthesis denotes enzyme activity as % of control.

Table-4. Acid phosphatase activity [µ moles (p-nitro phenol produced) seed^{-1} min^{-1}] in the embryo axis and cotyledons of the pea seeds germinated in presence of cadmium.

Treatment	Embryonic Axis				Cotyledons			
	2nd Day	4th Day	6th Day	8th Day	2nd Day	4th Day	6th Day	8th day
Control	7.68±0.29 (100)	14.22±0.54(100)	20.54±0.54(100)	25.09±0.04(100)	4.68±0.12 (100)	9.12±0.25 (100)	13.61±0.35(100)	15.93±0.54(100)
1 mM	5.66±0.27 (74)	11.48±0.8 (80)	14.63±0.57(71)	14.02±0.65(56)	3.12±0.54 (67)	6.78±0.54 (74)	9.33±0.36 (69)	11.34±0.41 (71)
2 mM	3.49±0.74 (45)	5.92±0.74 (42)	6.88±0.25 (33)	6.94±0.25 (28)	2.24±0.65 (48)	3.48±0.92 (42)	4.69±0.56 (34)	5.42±0.24 (34)
3 mM	2.94±0.54 (38)	4.01±0.85 (28)	4.69±0.65 (23)	5.03±0.23 (20)	1.91±0.21 (41)	2.87±0.56 (24)	3.22±0.23 (24)	3.94±0.54 (25)

Mean of three replicates ± S.D; Values in parenthesis denotes enzyme activity as % of control.

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Table-5. Peroxidase activity [μ moles (H_2O_2 consumed) $seed^{-1}$ min^{-1}] in the embryo axis and cotyledons of the pea seeds germinated in presence of cadmium.

Treatment	Embryonic Axis				Cotyledons			
	2nd Day	4th Day	6th Day	8th Day	2nd Day	4th Day	6th Day	8th day
Control	3.78±0.56 (100)	7.13±0.62 (100)	5.53±0.68 (100)	5.01±0.42 (100)	3.15±0.42 (100)	5.67±0.68 (100)	5.01±0.46 (100)	5.09±0.51 (100)
1 mM Cd	3.67±0.31 (97)	6.78±0.29 (95)	5.14±0.19 (93)	4.98±0.16 (99)	3.28±0.21 (104)	5.71±0.32 (101)	4.89±0.26 (98)	5.30±0.30 (104)
2 mM cd2	3.88±0.14 (103)	7.56±0.22 (106)	5.71±0.12 (103)	5.11±0.09 (102)	3.40±0.18 (108)	5.99±0.25 (106)	5.81±0.02 (115)	5.92±0.11 (116)
3 mM cd2+	4.08±0.32 (108)	7.82±0.56 (110)	5.90±0.42 (105)	5.86±0.30 (117)	3.48±0.22 (110)	6.00±0.19 (106)	6.08±0.22 (121)	6.28±0.26 (123)

Mean of three replicates ± S.D; Values in parenthesis denotes enzyme activity as % of control.

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