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Screening of Pea Genotypes for Cd^{2+} Tolerance on the Basis of Reproductive Behavior

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Abstract

Addition of 25 µM Cd^{2+} to the basal germination medium did not affect pollen germination except in HFP-8712, while higher doses exercised an inhibitory effect, and inhibition increased with the progressive increase in the level of cadmium. Among various genotypes, HFP-9426 showed minimum inhibition followed by HFP-0143, while maximum in HFP-2005 followed by HFP-9907A. Low level of $Cd2^{2+}$ (25 µM) stimulated tube growth in HFP-8712, HFP-8909 and HFP-9426, while higher concentrations were inhibitory. Maximum reduction in tube length was observed in Arkel and minimum in HFP-0143 at 500 µM Cd^{2+}. Pollen grains failed to germinate on medium supplemented with 1000 µM Cd^{2+}.

Introduction

Deployment of municipal based composts as fertilizer and irrigation of crop fields with sewer water constitute major strategies adopted by vegetable growers and marginal farmers. Though this strategy is intended to increase the fertility of soil and to circumvent scarcity of irrigation water, it inadvertently leads to addition of huge quantities of heavy metals (HMs) to the agro-ecosystem, which results into the deteriorated soil quality. Heavy metal problem has further been aggravated by various developmental and economic activities, such as rapid proliferation of industries, transport systems etc. These anthropogenic activities though intrinsically aimed at promoting the welfare of human race have resulted in an injudicious release in disconcerting large quantities of hazardous chemicals, including HMs into the biosphere. As the HMs is quite readily taken up by the plants and at the same time is non-degradable, there is great scope of their entry into the food chain. Being toxic, their excessive or continued intake, even in small amounts over a long period poses hazard for human and animal health [1,2]. The fatal consequences of ingestion of food, contaminated either accidentally or naturally with cadmium, are well documented by the incidence of "Itai-Itai" and "Miniamata" diseases in Japan [3]. Since HMs can be easily accumulated by agricultural crops, these inhibit plant growth and mineral nutrition.

Among different HMs, Cd^{2+} is of great concern as it is one of the most toxic metal pollutants of soil [4]. Slowly, the concentration of Cd^{2+} builds up in the soil and affects plant growth, photosynthesis, respiration and other metabolic processes, and finally economic yield [5-14]. Cadmium not only affects economic yield quantitatively, but also deteriorates its quality as well.

Although, there is preponderance of literature on physiological and biochemical aspects of HM toxicity, little is known with regard to Cd^{2+} effects on reproductive functions of plants, which bear direct relevance to seed setting and its maturation. Any deviation and distraction from normal differentiation and functioning of reproductive structures and sexual units is bound to affect crop yield adversely [15,16]. Being sedentary, plants have acquired or evolved a wide range of Cd^{2+} tolerance mechanism [7], which is restricted not only in different species, but also among the varieties/genotypes within the species [17].

Pulses including pea constitute an important source of poor man's dietary proteins. Pea (*Pisum sativum* L.) is one of the most important grain legumes, grown for its green pods as well as pulse crop. It is strictly self-pollinating vegetable crop.

There are evidences of existence of intraspecific genetic variations in the tolerance of legumes to HMs. For instance, cultivars of navy beans differ in Zn and Cu tolerance [18], while those of soybean and cowpea differed in Zn and Mn tolerance, respectively [19,20].

This warrants meticulous efforts for screening of these available diverse genotypes. Most of the screening procedures involve evaluation on the basis of germination and subsequent seedling growth, which is no way related to the final crop yield and hence, is not of pragmatic value. Therefore, it warrants having evaluation on the basis of one or more methods which have direct relevance to seed yield, or could indirectly indicate for the potential yield that could be harvested. Among an array of available methods, *in vitro* pollen germination assay, membrane injury, chlorophyll stability index and *in vitro* tissue culture responses in the presence of pollutant are anticipated to prove their legitimacy for screening against Cd^{2+} tolerance, keeping seed germination and early seedling growth as a check.

Induction of tolerance customary to HMs is an alternative approach to combat the problem of HM pollutants of soils. Conventional plant breeding has, however, failed to provide the desired results. Recently, it has been shown that 60-80% of the genes expressed in pollen [21,22] are also expressed in the sporophyte and consequently, correspondence in the behaviour of pollen and sporophyte to abiotic (Cu in *Mimulus guttatus*, herbicide like Alachlor, chlorosulfuran [23-25], and biotic stress (pathotoxin of *Heliminthosporium maydis* in corn) has been reported [26]. All these studies indicate that the application of selection pressure (stress) at pre-or post-pollination stage, which would eliminate stress sensitive pollen, may help in inducing stress tolerance. Present study, therefore, was undertaken to screen different pea genotypes for Cd^{2+} tolerance by pollen germination and pollen tube growth.

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Materials and Methods

The seeds of eleven genotypes of *Pisum sativum* i.e. Hisar Harit (PH-1), Arkel, HFP-2005, HFP-9907A, HFP-8712 (Jayanti), HFP-4 (Aparna), HFP-8909 (Uttara), HFP-9426 (Parvati), HFP-0143, HFP-0106 and HFP-0128 (Plate 1 and 2) were procured from the Sectional Head Pulses Section, Department of Plant Breeding, CCS Haryana Agricultural University, Hisar. For seed inoculation, *Rhizobium leguminosarum* strain *bv. viciae* was obtained from the Incharge, Biofertilizer Unit, Department of Microbiology, CCS Haryana Agricultural University, Hisar.

Experiment I: To screen different pea genotypes for Cd^{2+} tolerance by pollen germination and tube length method.

The experiment was aimed at investigating the relative tolerance status of different pea genotypes to Cd^{2+} by employing pollen germination and tube length method.

Chemicals and reagents: The chemicals and reagents used during the present investigation were of analytical grade.

Pollen performance: This was done to investigate relative tolerance status of different pea genotypes to cadmium, based on *in vitro* pollen germination and tube growth.

Growth conditions: Seeds were surface sterilized with 0.8% sodium hypochlorite solution for 8 min, to which one to two drops of teepol were added. Seeds were shaken regularly and then washed thoroughly with distilled water (3-4 times). These seeds inoculated with *Rhizobium leguminosarum* strain *bv. viciae* were finally sown in cement pots in the screen house. Each pot was lined with a polythene bag with a central drainage hole, and filled with 6 kg of acid treated and thoroughly washed river sand. Five seeds per pot were sown. Thinning was carried out after 20 days of sowing to leave three plants of uniform size in each pot. Plants were supplied with N-free nutrient solution at an interval of 10 days throughout the course of crop growth, except a starter dose of NO_3-N (45 mg/pot) [27]. Plants of each genotype were exposed to a range of $CdCl_2.2H_2O$ concentrations i.e. 0, 2.5, 5.0 and 7.5 mM, 15 days prior to anticipated date of initiation of flowering. Twenty pots per treatment were used in each genotype.

Amount of $CdCl_2.2H_2O$ given to each pot for a range of cadmium concentration is as follows:

i) Control	No Cadmium
ii) 2.5 mM	0.26 g/pot
iii) 5.0 mM	0.51 g/pot
iv) 7.5 mM	0.77 g/pot

Irrigation: Intermittent canal water irrigation was also given, as and when required.

***In vitro* pollen germination and tube growth:** Flowers were collected from three randomly selected plants, a day before anthesis from each genotype. The pollen from these flowers were mixed thoroughly on a glazed paper and sprinkled with the help of camel hair brush on the semi-solid medium contained in petriplates. The composition of the germinating medium was:

Sucrose=15%

Calcium nitrate=200 ppm (0.02%)

Boric acid=100 ppm (0.01%)

Agar=0.8%

Petriplates were incubated at 25 ± 2°C for 3 h in dark. Three petriplates per treatment were used. After incubation, pollen activity was terminated by flooding the surfaces of the medium with killing and fixing solution of following composition [28].

Formaldehyde=5 ml

Glacial acetic acid=3 ml

Water=72 ml

Glycerine=20 ml

Safranin (aq.) =2 drops (1%)

The petriplates were stored in a refrigerator at 8-10°C, until the observations were recorded. Pollen producing a tube length of a size greater than its diameter was designated as germinated. Ten readings for pollen germination and 30 for tube length from different microscopic fields of each petriplate were made from areas, with uniform distribution of pollen and fairly good populations. Pollen tube length was recorded in such a way that it presented an average of approximately 80 per cent of pollen tubes.

Experiment II: To study the effect of Cd^{2+} on reproductive behaviour of sensitive and tolerant pea genotypes

Based on the results of the experiment I, two genotypes - one relatively sensitive (HFP-0106) and relatively tolerant (HFP-0143) genotypes were selected and raised in the screen houses of Department of Botany and Plant Physiology, CCS Haryana Agricultural University, Hisar. Seeds were surface sterilized with 0.8% sodium hypochlorite solution for 8 min, to which one to two drops of teepol (detergent) were added. Seeds were shaken at regular intervals. After surface sterilization, seeds were washed thoroughly with distilled water (3-4 times) and were inoculated in a broth of *Rhizobium leguminosarum* strain *bv. viciae* for half an hour, and finally sown in cement pots. Each pot was lined with a polythene bag with a central drainage hole, and filled with 6 kg of acid treated and thoroughly washed river sand.

Five seeds were sown in each pot. Thinning was carried out after 20 days of sowing to leave three plants of uniform size in each pot. Plants were supplied with N-free nutrient solution at an interval of 10 days throughout the course of crop growth, except for the starter dose of NO_3-N (45 mg/pot) [27].

Composition of nutrient solution stock

Major salts	**Quantity (g/l)**
1. $CaCl_2.2H_2O$	94.4
2. KH_2PO_4	108.0
3. $MgSO_4.7H_2O$	106.0
4. K_2SO_4	138.6
5. **Minor salts**	**(mg/l)**
i) $CuSO_4.5H_2O$	124
ii) $MnCl_2.4H_2O$	400
iii) $ZnSO_4.7H_2O$	575
iv) H_3BO_3	1537
v) Na_2MoO_4	100
vi) $CoCl_2$	45
6. Ferric citrate	1 mg/ml

The nutrient solution for final use was prepared by adding 40 ml of each of the first five stock solutions and 16 ml of sixth solution, and volume was made to 16 litres with canal water. Plants of both the genotypes were exposed to a range of cadmium chloride (control, 2.5, 5.0 and 7.5 mM) treatment, nearly 15 days prior to anticipated date of flowering. Twenty pots per treatment were used for each variety.

Intermittent canal water irrigation was also given, as and when required. With the commencement of flowering, following parameters were recorded.

1. Date of appearance of first flowering bud
2. First flowering bud node
3. Total flowering nodes
4. Number of flowers at each node
5. Total flowering buds
6. Total number of flowers per plant: It was recorded by counting the number of flowers produced every alternate day, until the flowering was complete.
7. Fresh and dry weight of flower: Flowers were taken at the day of anthesis, weighed and kept for drying in the forced air oven at 80°C, until the constant weights were obtained.
8. Male fitness characteristics: Following characteristics were recorded:

   i. Numerical production of pollen: Floral buds were collected a day before anther dehiscence, and employed for quantification of pollen grains produced per flower. Number of pollen grains was calculated by suspending pollen grains of ten anthers in 2 ml of 50 per cent glycerine, containing few drops of safranine solution. Glass rod was used to crush the anthers and thus, suspension obtained was passed through a brass sieve, with a mesh of 48 sq/cm^2 [29]. Number of pollen grains per drop was counted by placing it on clean and dry microslide. Pollen grains per flower were computed by counting ten drops of pollen suspension per replicate, and three replicates per treatment were used.

   ii. Pollen diameter: Floral buds were collected at anther dehiscence stage from three randomly selected plants, and pollen of these floral buds were suspended in 50% glycerine for 30 min and used for measuring pollen diameter under a light microscope. Three replicates were used for each treatment.

   iii. Pollen viability: Viability of freshly released pollen grains was assessed by 2,3,5-triphenyl tetrazolium chloride (TTC) test [30].

Reagents: a) TTC solution: 0.5% TTC (w/v) in 15% sucrose

b) Sodium succinate crystals

Procedure: To the TTC solution taken in a test tube, few crystals of sodium succinate were added. A drop of this solution was put on a clean and dry microslide and small amount of pollen was sprinkled over TTC drop, and coverslip was applied immediately. It was incubated at 35-40°C for 5 minutes in dark. At the end of the incubation period, preparations were scored for percentage of viable pollen grains (bright red) under a light microscope. Ten observations per replicate and three replicates per treatment were taken for this test. Percentage pollen viability was computed from this data.

i) *In vitro* pollen germination and tube growth: Flowers were collected from three randomly selected plants, a day before anthesis from each treatment. The pollen from these flowers were mixed thoroughly on a glazed paper and sprinkled with the help of camel hair brush on the semi-solid medium contained in petriplates. Petriplates were incubated at 25 ± 2°C for 3 h in dark. Three petriplates per treatment were used. After incubation, pollen activity was terminated by flooding the surfaces of the medium with killing and fixing solution [28]. Pollen germination (%) and tube length was recorded as described earlier.

Statistical analysis: The data was analysed using analysis of variance for the complete randomized design (CRD), where each observation was replicated thrice. Coefficient of concordance and Spearman's Rank Correlation Analysis was also done.

Results

Pollen germination

Addition of 25 µM Cd^{2+} to the germination medium did not affect pollen germination except HFP-8712, while higher doses exercised an inhibitory effect on pollen germination and inhibition increased with the progressive increase in the level of cadmium. Among various genotypes, HFP-9426 (Plate 3) showed minimum inhibition followed by HFP-0143 (Plate 4), while germination was inhibited maximally in HFP-2005 (Plate 5) followed by HFP-9907A (Table 1).

Pollen tube growth

Pollen grains of HFP-0143, HFP-9426 and HFP-4 produced longest pollen tubes, while those of HFP-2005, Hisar Harit had the shortest ones. The addition of low level of Cd^{2+} (25 µM) to the basal germination medium stimulated pollen tube growth in HFP-8712, HFP-8909 and HFP-9426, while higher concentrations were inhibitory (Table 2). In other tested genotypes, pollen tube length decreased significantly with the increase in level of cadmium. At 500 µM Cd^{2+}, maximum reduction in tube length was observed in Arkel (Plate 6) and HFP-4, while reduction was at minimum in HFP-0143 and HFP-9426. Pollen grains failed to produce pollen tube when treated with 1000 µM Cd^{2+} (Plate 7 and 8).

Discussion

In vitro studies have revealed that pollen germination decreased with increasing level of Cd^{2+} in the germination medium. Among various genotypes, HFP-9426 showed minimum inhibition followed by HFP 0143, while it was inhibited maximally in HFP-2005 followed by HFP-9907A. Low concentration (25 µM) of cadmium stimulated tube growth in Hisar Harit, HFP-2005, HFP-8712, HFP-8909, HFP-9426, HFP 0106 and HFP-0128, while higher concentrations were inhibitory. Stimulation of tube growth by lower dose of Cd^{2+} (2.5 mM) in HFP-4 and HFP-4 (x) Arkel [12], and Ni in *Datura* and tomato are well documented [31]. At 500 µM, maximum reduction in tube length was evident in Arkel and HFP-4, and minimum in HFP-0143 and HFP-9426. Pollen grains of all the tested genotypes failed to produce a tube, on a medium containing 1000 µM Cd^{2+}. Inhibitory effect of HMs on pollen germination and tube growth in corn [32], *Vicia tetrasperma* [33], *Malus sylvestris* [34], *Ecalyptus tereticornis* [35], poppy, *datura* and tomato [31] are widely documented.

Sensitivity of pollen grains of Arkel to Cd^{2+} has been reported [15,12]. Similarly, Varshney and Chauhan [36] also reported that

Genotype	Cd^{2+} treatment (µM)				Mean
	Control	25	50	500	
Hisar Harit (PH-1)	59.43	56.25 (-5.35)	53.76 (-9.54)	47.92 (-19.36)	**54.34**
Arkel	65.69	61.02 (-7.10)	55.25 (-15.88)	49.80 (-24.18)	**57.94**
HFP-2005	52.20	56.49 (+8.21)	51.76 (-0.84)	31.14 (-40.34)	**47.90**
HFP-9907A	58.98	53.88 (-8.64)	47.00 (-20.31)	37.18 (-36.96)	**49.26**
HFP-8712 (Jayanti)	65.64	55.66 (-15.20)	53.63 (-18.29)	47.24 (-28.03)	**55.54**
HFP-4 (Aparna)	63.61	60.79 (-4.43)	54.58 (-14.19)	48.76 (-23.34)	**56.93**
HFP-8909 (Uttara)	64.04	61.93 (-3.29)	54.51 (-14.88)	52.82 (-17.52)	**58.33**
HFP-9426 (Parvati)	64.19	63.28 (-1.41)	62.61 (-2.46)	59.53 (-7.25)	**62.40**
HFP-0143	67.39	63.21 (-6.20)	60.70 (-9.92)	57.88 (-14.11)	**62.30**
HFP-0106	70.89	67.17 (-5.24)	61.51 (-13.23)	46.81 (-33.96)	**61.60**
HFP-0128	69.80	64.23 (-7.97)	68.06 (-2.49)	47.92 (-31.34)	**62.50**
Mean	**63.81**	**60.36**	**56.67**	**47.91**	

Figures in parentheses indicate per cent change over respective controls
CD at 5% LS
Genotype (G) = 2.66
Treatment (T) = 1.61
GxT = 5.33

Table 1: Effect of Cd^{2+} added to the basal germination medium, on per cent *in vitro* pollen germination (Arcsin transformed mean) in different genotypes of pea.

Genotype	Cd^{2+} treatment (µM)				Mean
	Control	25	50	500	
Hisar Harit (PH-1)	200.25	240.38 (+20.04)	167.83 (-16.18)	53.76 (-73.15)	**165.55**
Arkel	278.78	182.61 (-34.49)	142.96 (-48.71)	39.56 (-85.80)	**160.98**
HFP-2005	171.06	197.28 (+15.32)	139.06 (-18.71)	37.05 (-78.34)	**136.11**
HFP-9907A	370.57	246.60 (-33.45)	176.77 (-52.29)	93.09 (-74.87)	**221.76**
HFP-8712 (Jayanti)	361.23	425.89 (+11.71)	337.81 (-11.38)	118.55 (-68.90)	**315.87**
HFP-4 (Aparna)	409.23	409.00 (-0.05)	142.25 (-65.24)	65.38 (-84.02)	**256.47**
HFP-8909 (Uttara)	368.72	480.6 (+30.36)	272.16 (-26.18)	81.07 (-78.01)	**300.65**
HFP-9426 (Parvati)	469.21	619.28 (+31.98)	346.41 (-26.17)	213.38 (-54.52)	**412.07**
HFP-0143	475.29	411.89 (-13.33)	225.87 (-52.47)	241.24 (-49.24)	**338.58**
HFP-0106	250.35	282.81 (+12.96)	196.51 (-21.50)	82.12 (-67.19)	**202.95**
HFP-0128	297.26	354.58 (+19.28)	361.49 (-28.33)	124.00 (-58.28)	**289.33**
Mean	**333.81**	**350.09**	**229.92**	**104.47**	

Figures in parentheses indicate per cent change over respective controls
CD at 5% LS
Genotype (G) = 31.93
Treatment (T) = 19.25

Table 2: Effect of Cd^{2+} added to the basal germination medium, on *in vitro* pollen tube length (µm) of different genotypes of pea.

pollen of Arkel and Bonnerville genotypes were sensitive to Cr (VI); HFP-4 being most tolerant, while those of HFP-8712, Hisar Harit and Rachna were the moderately tolerant. Raj et al. [15] reported that pollen germination and tube growth declined significantly in ten cultivars of pea with increasing level of Cd^{2+} and at higher concentrations, germination was completely inhibited in PH-1, Arkel, VRP-3 and Rachna. Similarly, pollen germination has been reported to decrease in *Mimulus guttatus* with the progressive increase in the concentration of copper, and germination was completely inhibited at 10 µM in sensitive clones, while those from tolerant attained a peak in pollen germination and tube growth at 10 µM [37], thereby suggesting that metal tolerance is correlated with pollen source. This is further ascertained from the studies showing that metal sensitive plants of *Silene dioica* and *S. alba* produced pollen with more sensitivity to Cu and Zn than tolerant ones. Pollen of *Viola calaminaria* had an absolute requirement of Zn [37].

Cadmium mediated reduction in tube length may be ascribed to its delayed emergence, as has been reported in Impatiens balsamina [38] and metal toxicity on tube growth. Cadmium mediated toxicity may result from the binding of Cd to –SH groups in proteins, leading to an inhibition of activity or disruption of structure, or from the displacing of an essential element resulting in deficiency effects [39]. In addition, Cd^{2+} excess may stimulate the formation of free radicals and reactive oxygen species resulting in oxidation stress [40]. In addition, metabolic disorders induced by Cd^{2+}, it may have direct effect on growth e.g. due to interactions with cell wall polysaccharides, thus decreasing cell wall plasticity.

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