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RESPONSE OF CADAVERINE ON THE CULTURED TISSUES OF *BRASSICA JUNCEA* (RH-30)

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ABSTRACT

Cadaverine (Cad), out of two diamine of growth modulating polyamines which is characteristics synthesize entirely from different pathway with respect to the group, anticipated in growth of the plant, but antistress role is obscure. The Cad effect on germination and plant growth under various stress regimes (from explants hypocotyls of Indian mustard *Brassica juncea* RH-30) The tissue culture media was MS induced with metal Cd and Pb (1mM) and NaCl salinity was 100mM. The explants under Cad media showed better growth over control and stressed one. Further, subculturing of callus in the presence of Cad maintained high callus growth over control and even with NH_4NO_3 supplementation. However, Cad did not check the inhibitory effect of salinity and Pb stress on sub-cultured callus growth. On the other side supplementation of Cad did not show any stimulatory effect on nitrate reductase activity except that a little increases in activity with salt and Pb presence. Cad ameliorates the growth of the callus under stress conditions and these stress induced proteins in the presence of Cad may help plant to grow under stress condition.

KEYWORDS: *Brassica juncea*, Cadaverine, Metal, Salinity, Stress.

INTRODUCTION

B. juncea is valued for its intense flavours and healing properties. This plant is cultivated mainly as an oil crop. It is a good bee plant.[1] All over the world, mustard is used for its appetizing flavor and preservative value and the seeds are used largely for tempering food. Mustard is available in the form of seeds, powders and oil. Recently, *B. juncea* has been explored for its biodiesel potential.[2] The cadaverine (Cad), structurally different diamine and having independent biosynthetic pathway in relation to putrescine (Put), also grouped in the family of polyamines regarded as a growth regulator [3] and proposed same mode of action. [4] The Cad also tends to accumulate in higher plants under osmotic/salt stresses [5] like Put, but without explicit explanation of its role in specific stress alleviation. In view of some conflicting observations it is pertinent to peruse that Cad inspite of structural difference with that of Put or ammonium nitrate might pose some different responses in plants under different conditions. It has been observed that salinity has no relationship with Cad level in *Oryza sativa*[6] While, there is either little change in maize[7] or decrease in wheat, or no accumulation in barley and *Vicia faba.*[8,9] Aziz et al.[10,11] have demonstrated Cad accumulation in osmotically stressed leaf disc of rape and tomato under salinity.

Carrizo et al. [12] have demonstrated age dependent Cad titer, which declined progressively in *Brugmansia candida*. The polyamines in general have been assigned as an antisenescence molecule [5,10,13]along with Put [14,15]. However, Cad is implicated in differential alkaloid synthesis in plants. [12] Few biological role of Cad during oxidative stress is demonstrated recently. [13,16,17] Thus, the Cad *in vitro* application on callus under stress likely to exert some positive response in stressed plants, which is not elucidated well. Therefore, the present study may provide some insight to understand its some regulatory mechanism in plants growth and its potential to mitigate the adverse responses induced under multiple stresses, generally realized by plants in field condition vis-a–vis to compare its response with NH_4NO_3.

MATERIAL AND METHODS

Seeds sampled for uniform size of similar morphology were sterilized by using 70% alcohol for 1 min and with 0.2% (w/v) mercuric chloride for 5 min and then thoroughly washed 5-6 times by sterile distilled water before planting and cultured on MS media. [18] The basal medium contained 3% sucrose and 0.7% agar-agar along with NaCl (100 mM), Cd or Pb (1mM) and added with cadaverine (Cad 1mM) or NH_4NO_3 (5mM) after autoclaving by using membrane filter of 0.2 micron. The pH 5.8 of the nutrient solution in all conditions of

treatments was kept constant. After 7 days the explant (hypocotyls) of the seedling were taken and cultured in MS media supplemented with different growth hormones (BAP 2.0 mg/l and NAA 0.2 mg/l) along with stress treatments. The explants were incubated at 25 ± 2^0 C, 65% RH for 3 weeks. After callus formation, the portion of the callus again incubated in the same condition as mentioned above.

The seed germination was counted following radicle and plumule emergence after 24 h and 48 h of sowing. The growth rate under stress is also determined by measuring fresh and dry wt. of callus after 3 and 4 weeks. Measurement of root, shoot length and fresh weight was done immediately after harvesting at 7^{th} day. Nitrate reductase activity (*in vivo*) was assayed by following the method of Srivastava [19] by using callus (.5 g). The data given are mean value of at least three replicates with ± SD. The student t-test was applied to find out the significance of the treatments.

RESULTS

Seed Germination, Shoot/Root Length, Fresh and Dry Weight

The seed germination was same almost in all the treatments either with NH_4NO_3 or with Cad. However, slightly reduced germination was found with Cad under NaCl stress (Fig. 1). Shoot length of the seedlings in the MS medium was almost same to NH_4NO_3 supplemented one. Though, NH_4NO_3 was showing very sharp decline in shoot length of the seedlings stressed with NaCl (100mM) or Pb (Fig. 2). While Cad inclusion in the medium increased the shoot length prominently compared to control or NH_4NO_3 treated one. However, Cad supplementation did not show same response in the presence of NaCl or Pb.

Moreover, it was noticed that NH_4NO_3 response on shoot length either under salinity or Pb stress was reversed by Cad application (Fig. 2). The root length in NH_4NO_3 supplemented seedlings was similar to control. Root length response was little deviated with that of the shoot of the seedlings under those stress conditions. NH_4NO_3 supplementation increased root length remarkably under saline condition while, decreased under Pb stress. Cad did not change the root length over control, rather caused decrease under salinity, while almost negligible effect was under Pb stress condition.

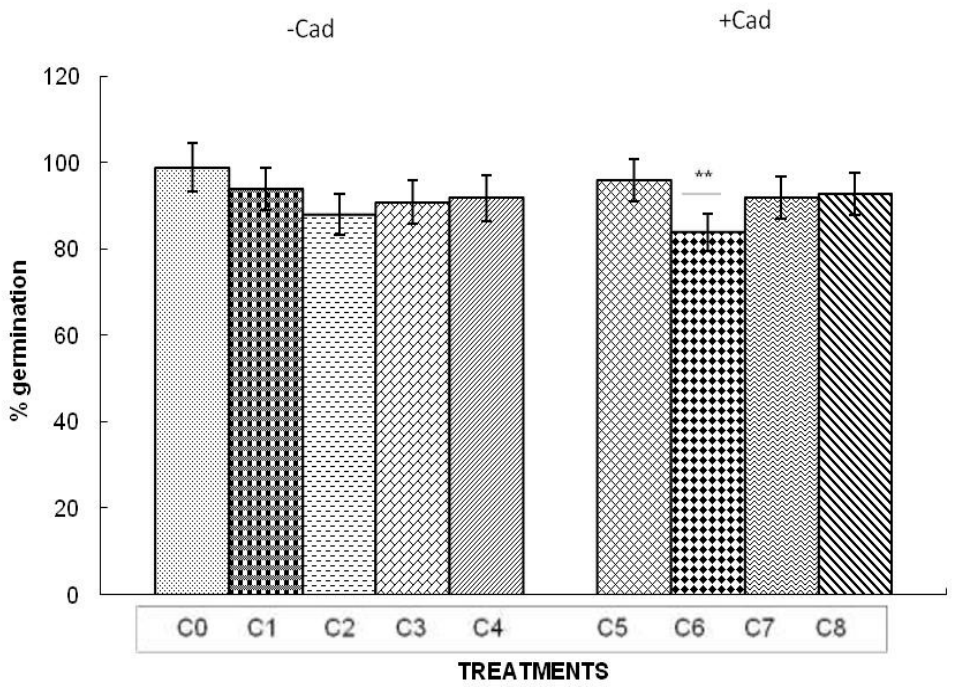
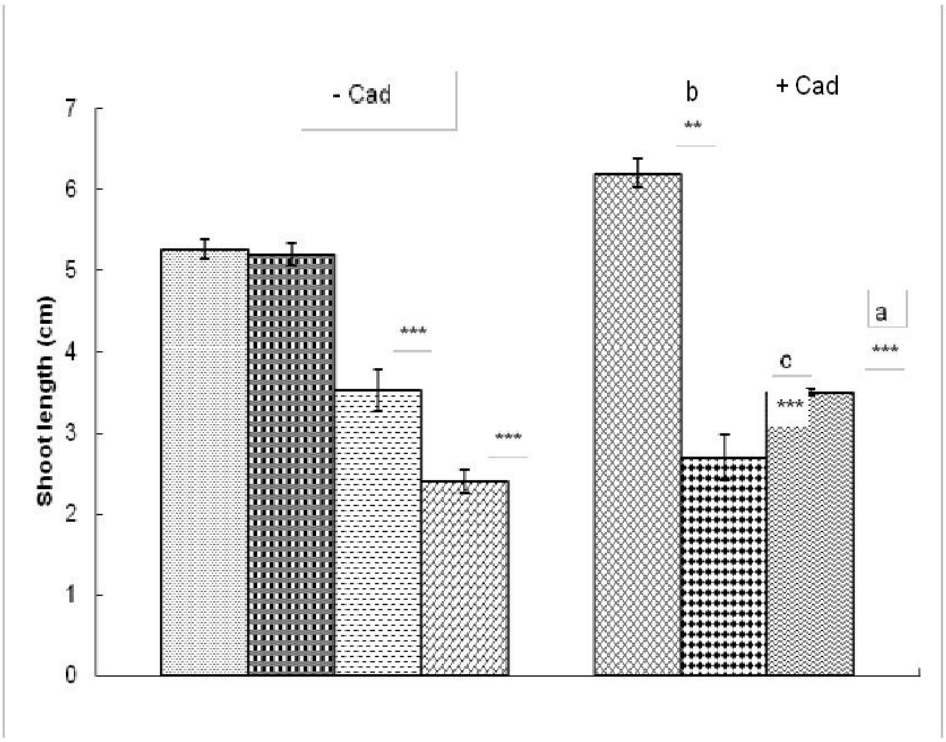


Fig. 1: Seed germination percentage in MS media included with either NaCl or Pb or Cd with NH_4NO_3 or Cad.

Data are mean value of replicas with (n=3) ± SD. Asterisks indicate the significance of difference at p<0.05% (*) probably significant, p<0.01 (**) definitely significant, p<0.001 (***) highly significant and No asterisks indicate insignificant p> = 0.05. Comparison between two nitrogenous source were indicated by p<0.05% (c) probably significant, p<0.01 (b) definitely significant, p<0.001 (a) highly significant and No symbol indicate insignificant p> = 0.05.

C0 - Control
C1 - NH_4NO_3 (5mM)
C2 - NH_4NO_3 + NaCl(100mM)
C3- NH4NO3 + Pb (1mM)
C4 -NH4NO3 + Cd (1mM)
C5 - Cad (1mM)
C6 - Cad+NaCl
C7 - Cad+Pb
C8 - Cad+Cd

Cad supplementation to growing seedlings showed less fresh and dry weight accumulation compared with that of NH_4NO_3 supplemented seedlings (Fig. 3). **Though seeds germination in the presence of Cd was almost same as in other stress conditions, but very-very poor growth of the seedlings in Cd-stress condition compelled not to perform any further experiments in *in vitro* with these tissues.**



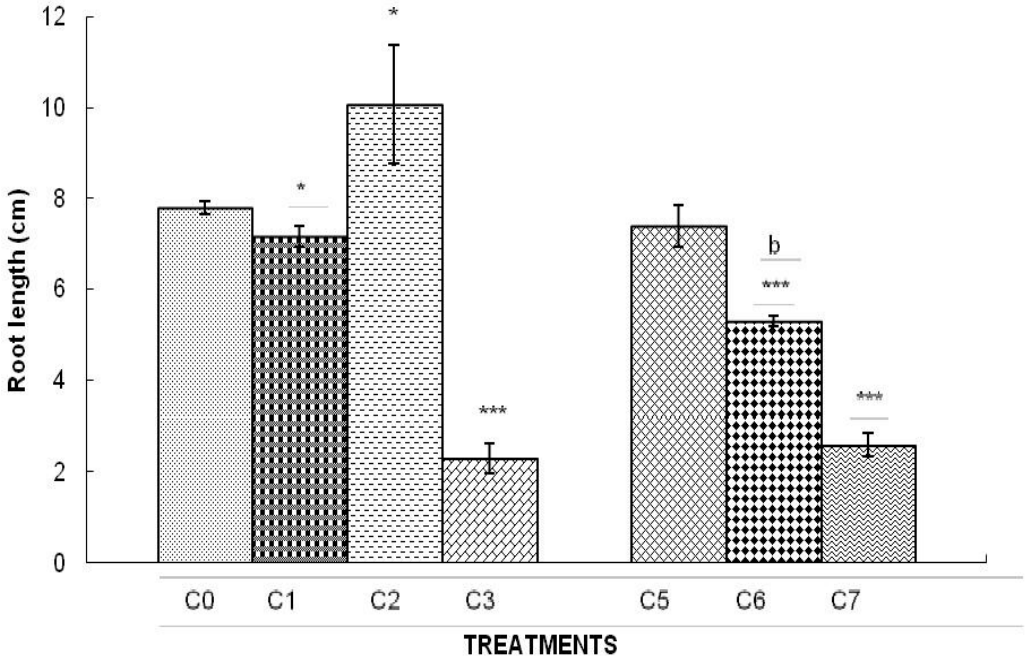


Fig. 2: Shoot and Root length of mustard seedlings in presence of NH_4NO_3 and Cad grown in MS media.

Rest legend same as Fig.1

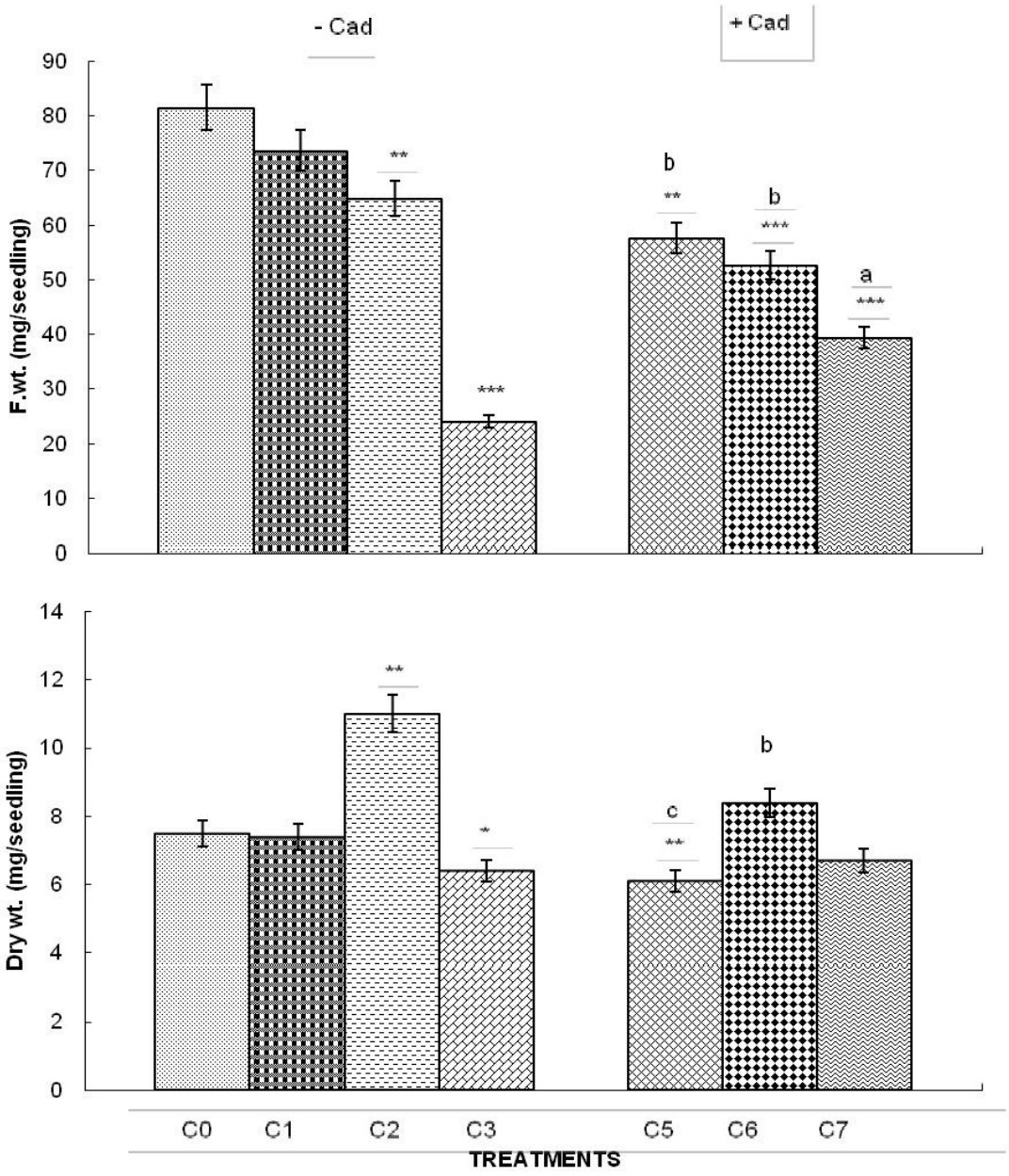


Fig. 3: F.wt./Dry wt. mustard seedlings in presence of NH_4NO_3 and Cad grown in MS media.

Rest legend same as Fig.1

Callus Growth under Stress in Presence and Absence of Cad

The callai from hypocotyls explants grown on MS medium supplemented with BAP 2.0 mg/l and NAA 0.2 mg/l for 3 weeks was remained undifferentiated under all kind of the stress condition (Plate 1). The NH_4NO_3 (5mM) inclusion in the medium reduced the callus fresh

weight drastically, which was further elevated under NaCl stress (Fig. 4; Plate 1). Infact, NH_4NO_3 was found stimulatory to callus growth under Pb (2mM) stress over control. On the other hand, the Cad inclusion in the MS medium increased two fold callus fresh weight over growth in the absence of Cad. Though, Cad inducing effect was not observed under salinity or Pb stress. The dry weight of callus was found more with Cad under salinity but not under Pb stress in comparison to NH_4NO_3 supplementation to the callus. The sub-culturing of these calluses under same condition showed further varied growth response (Fig. 5; Plate 2,3). The fresh and dry wt. was measured after 4 weeks. The callus growth was found decreased with NH_4NO_3 again here over control and the decrease continued further under salinity and Pb stress. But the Cad presence further maintained high callus growth over control and NH_4NO_3 supplemented callus. However, Cad did not check the inhibitory effect of salinity and Pb stress on sub-cultured callus growth. The dry wt. of sub-cultured callus was comparatively higher than that of Cad supplemented callus under the salinity stress condition, except that little more dry mass accumulation was found in Pb stressed callus.

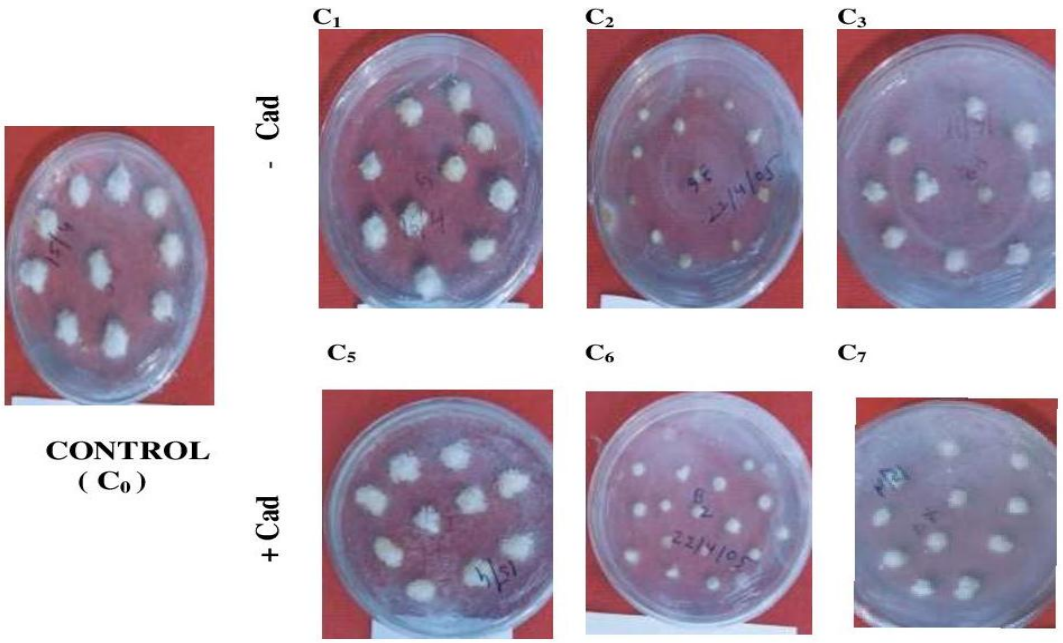


PLATE 1: Callus formation from hypocotyle explant after 3 weeks.

C0 - Control
C1 - NH_4NO_3 (5mM)
C2 - NH_4NO_3 + NaCl(100mM)
C3- NH4NO3 + Pb (1mM)
C4 -NH4NO3 + Cd (1mM)
C5 - Cad (1mM)
C6 - Cad+NaCl
C7 - Cad+Pb
C8 - Cad+Cd

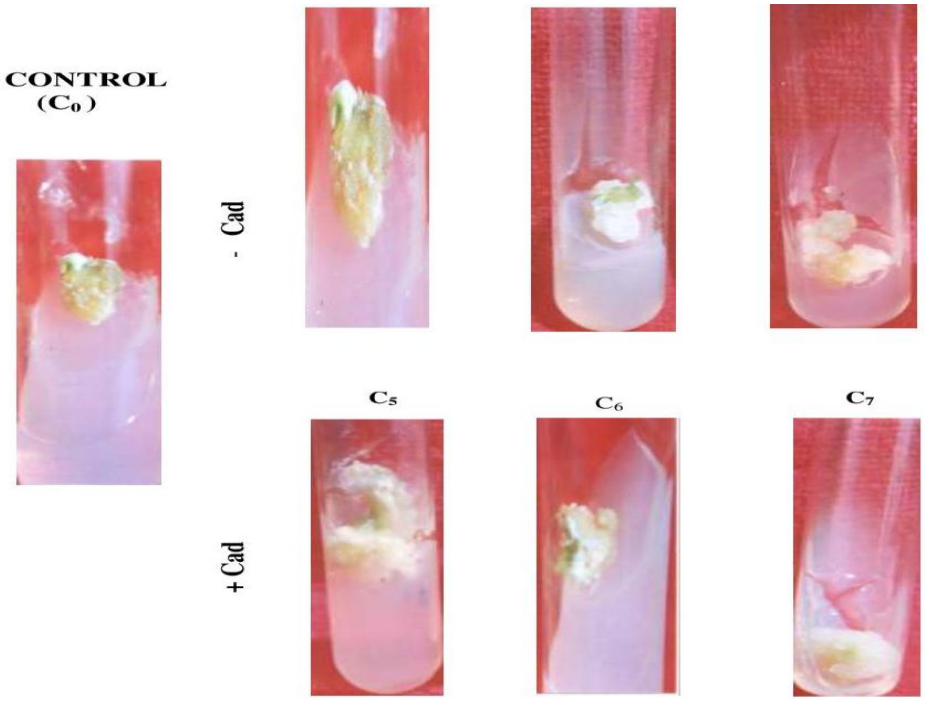


PLATE 2: Subculturing of Callus for shoot formation after 2 weeks.

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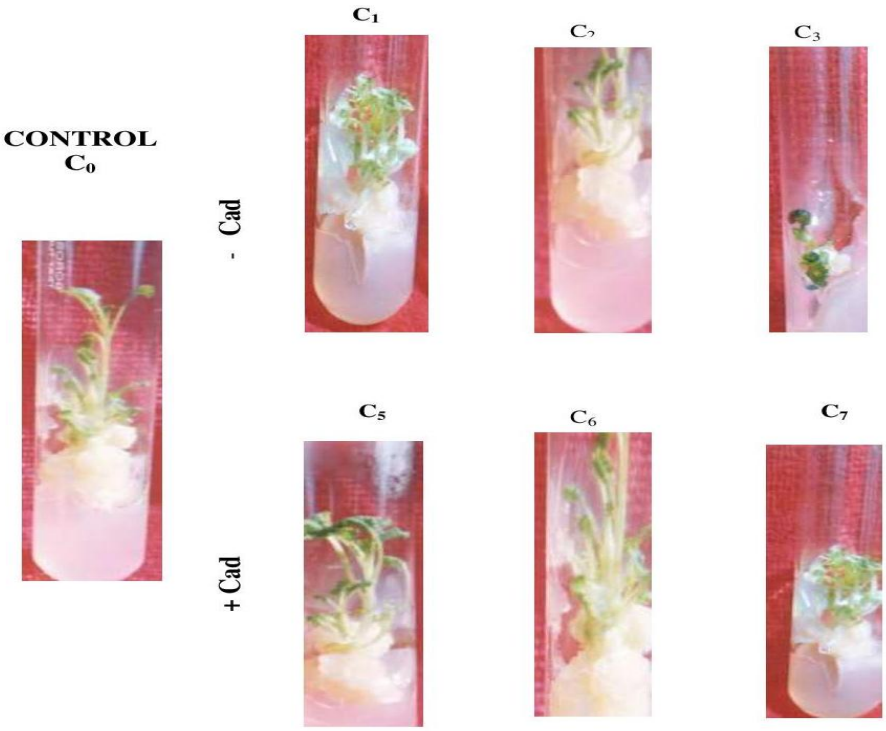

PLATE 3: Shoot formation after sub culturing of callus.

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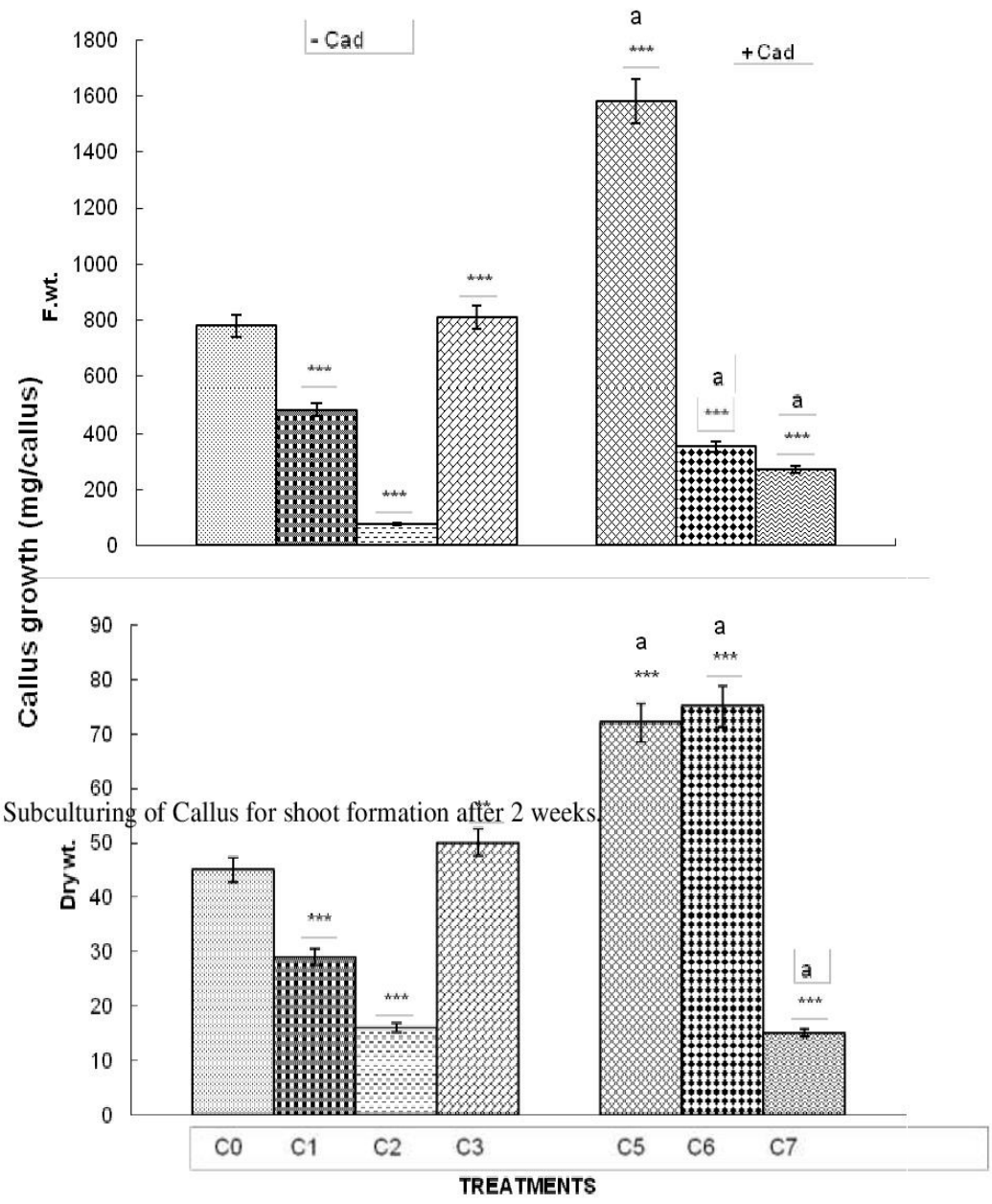

Subculturing of Callus for shoot formation after 2 weeks.

Fig. 4: Callus growth after 3 weeks in presence of NH_4NO_3 and Cad.

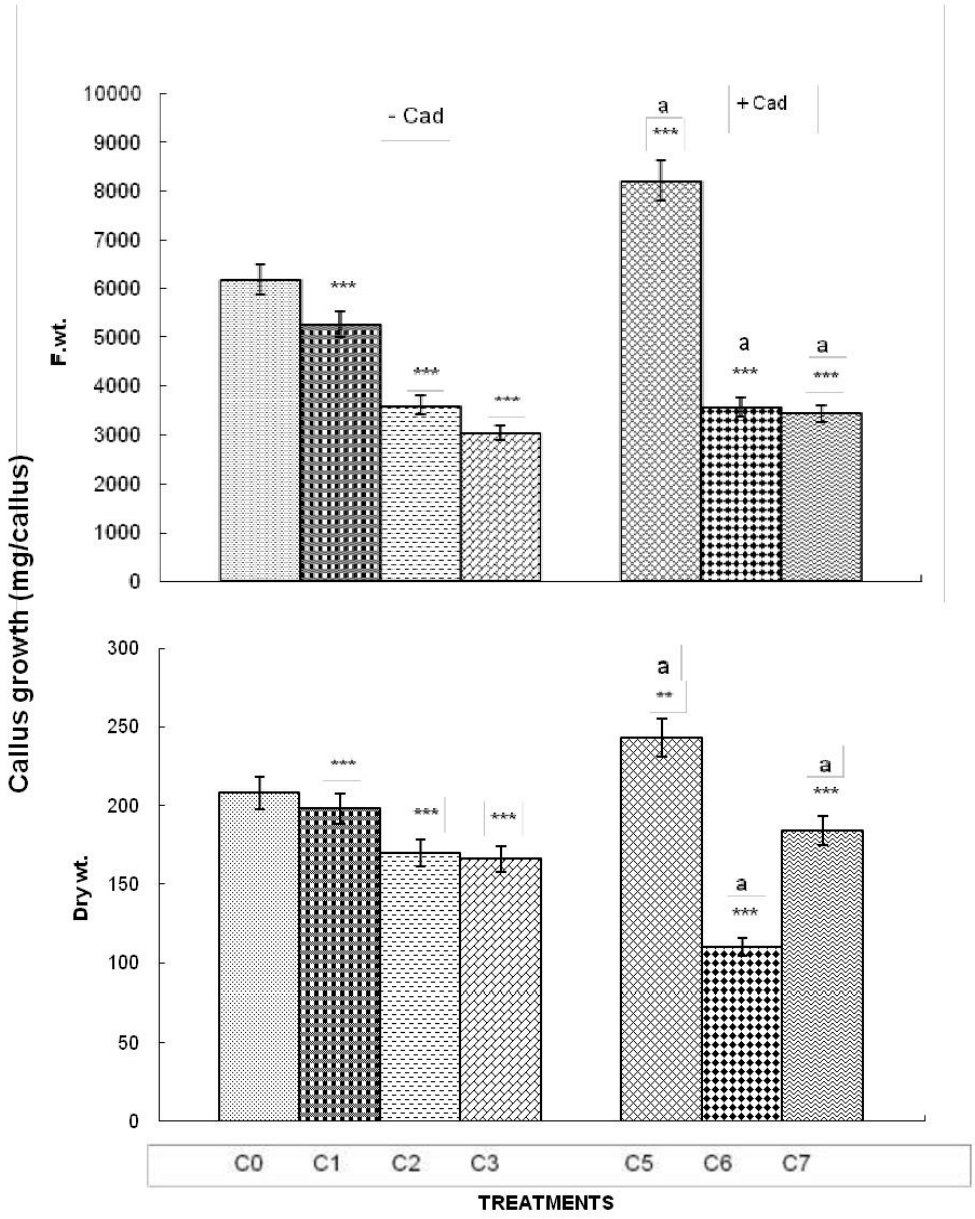

Fig. 5: Callus growth in subculture after 4 weeks in presence NH_4NO_3 and Cad.

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NRA in Growing Callus under Stress in Presence and Absence of Cad

The nitrate reductase activity (in *vivo*) examined in the growing callus after 3 weeks and after 4 weeks of sub-culturing of callus revealed that ammonium nitrate increased the nitrate activity in callus (Fig. 6A). The inorganic nitrogen was inductive of NRA in the callus growing on the Pb stress medium also, while little decrease was observed in the enzyme activity in the callus growing under salinity. Surprisingly, Cad supplementation in the medium inhibited the enzyme activity drastically. Callus stimulated enzyme activity was

almost one and half fold in the salt stressed callus and considerable under the Pb stress. The change pattern of this nitrogen assimilating enzyme was found all together changed in the sub cultured callus (Fig. 6B). Ammonium nitrate supplementation in the medium in the presence of NaCl or Pb stress also showed reduction in enzyme activity. The supplementation of Cad also did not show any stimulatory effect on enzyme activity except that a little increases in activity with salt and Pb presence.

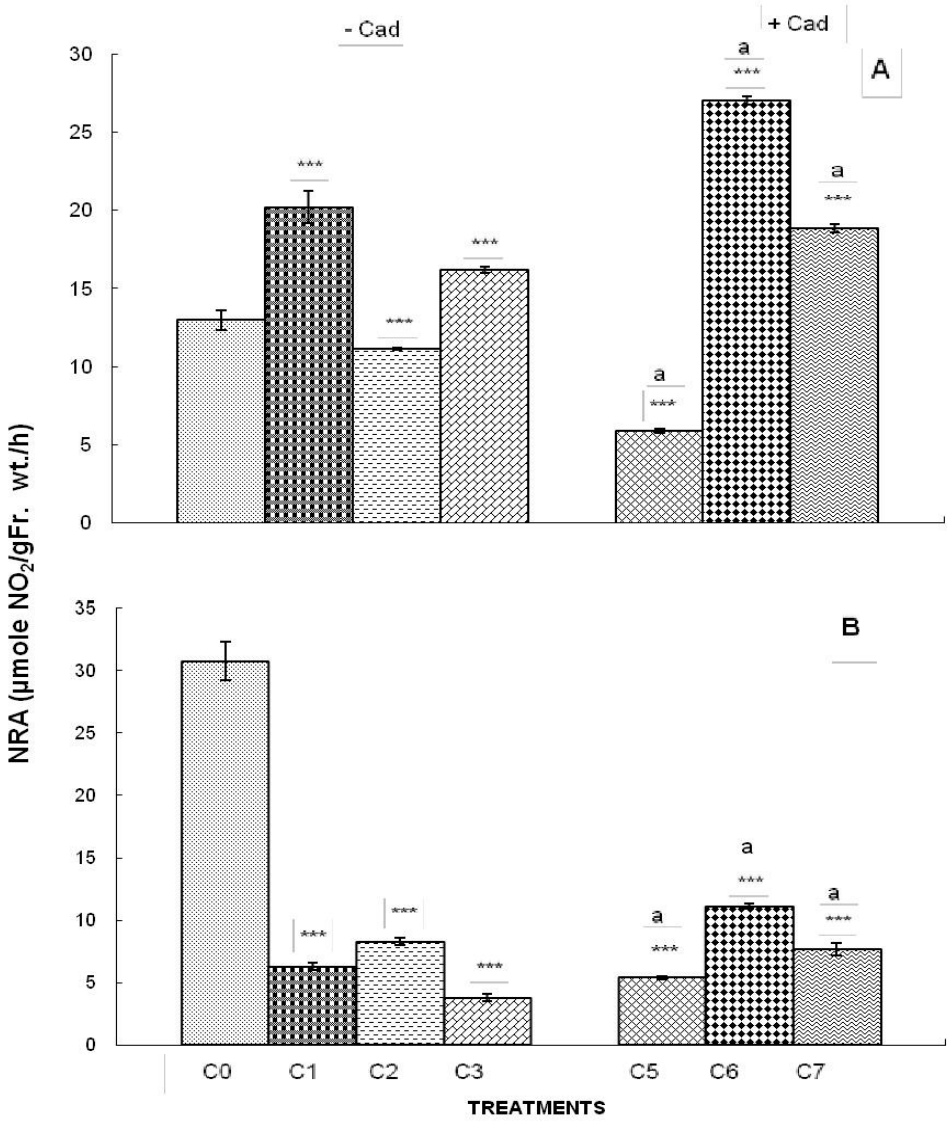

Fig. 6: NRA in presence of NH_4NO_3 and Cad in callus (A) and sub cultured callus (B) of explants from mustard seedlings.

Rest legend same as Fig.1

DISCUSSION AND CONCLUSION

The exogenous application of PA's, including diamines are able to retard the symptoms of senescence [20-22] with a view, the results of experiments here demonstrated that the Cadaverine has a potential to modify metabolites in growing cells. Over the last several decades, intensive research in plant cell and tissue culture through mainly manipulation of various biotic and abiotic factors affecting shoot organogenesis [23] has resulted in our better understanding in the physiological aspect of plant morphogenesis. Instead, the underlying mechanism of plant morphogenesis *in vitro* is not well understood. [24] The Cadaverine exhibits varied responses during callus growth and subcultured cell growth (Fig. 4,5; Plate 1) Chen et al. [25] observed that a medium containing the combination of 2 mg l-1 2,4-dichlorophenoxyacetic acid and 1 mg l-1 BAP significantly improved shoot regeneration in an anther culture of flax. In another study, the maximum shoot regeneration frequency in *Brassica* species was obtained in medium supplemented with 3.0 mg l-1 BAP and 0.15 mg l-1 NAA. [26] Hypocotyls of *Beta vulgaris* showed the best response of adventitious shoot regeneration in medium supplemented by BAP and NAA. [27] However, other studies have shown that shoot formation on hypocotyls of *Linum* seedlings was marginally promoted by a cytokinin (BAP) or thidiazuron. [28] In our system MS media supplemented with different growth hormones (BAP 2.0 mg/l and NAA 0.2 mg/l) along with cadaverine was taken for better growth.

No significant effect was observed with Cad in germination (Fig.1). In NaCl treated seeds with low germination were observed both with and without Cad. The seeds germination in the presence of Cd was almost 92 percent same as in other conditions, but thereafter poor growth of the seedlings even after supplementation of Cad/ NH_4NO_3 compelled not to perform any further experiments in *in vitro*.

Cad has shown tremendous growth in shoot length. This confirmed the Cad role in growth and development of seedlings (Fig. 2). NH_4NO_3 treated plants have better fresh biomass and dry mass per seedling (Fig. 3). The Cad increased the seedling water content, probably helping in maintaining osmotic and turgor pressure, in turn inducing tolerance, evident by increase in dry matter and to an extent elongation of root/shoot. The stress inhibition can be reverted by nitrogen presence in exogenous medium, and the better performance in term of organogenesis can be with Cad than NH_4NO_3.

The shoot formation (Fig. 1, 2) was observed in hypocotyls in accordance with other studies. [29] On hypocotyl explants of *Brassica napus* cv. Oscar cultured on 2,4-D-supplemented media, shoot regeneration was induced sporadically. Klimaszewska and Keller [30] reported that continuous culture on media supplemented with 2,4-D did not produce any shoot formation. The differences between their results and ours (Fig. 1, 2) may be attributable to differences between genotypes, but it is also possible that transferring hypocotyls to hormone-free medium is critical to shoot formation. Results obtained by Zheng and Konzak [31] in *Triticum aestivum* clearly showed that the continuous presence of a 2,4-D concentration that satisfied callus induction inhibited further development and subsequent plant regeneration.

The hypocotyls were used for callus generation (Plate 1-3). The hypocotyl-derived callus had better morphogenic ability. [25] Callus growth with salinity and metal stress was low when compared to the control. [32] The maximum growth was observed after 4 weeks of old callus in all the treatments treated with NH_4NO_3 as well as with Cad. In spite of lower growth of cells in saline medium, the tissues survived well up to 21 days in the respective medium (Plate 1-3). Gangopadhyay et al. [33] reported that cultured cells and/or callus of adapted glycophytes grew slower in medium containing NaCl than cells not adapted to salinity, growing without stress.

Ullah et al. [34] reported that on *Brassica napus* cv. Rainbow explants, callus proliferation started from the cut ends of the hypocotyl. High dry wt. was observed in Cad treated callus as compared to NH_4NO_3 treated one (Fig. 4). External stress can either result in an increase or decrease in cellular polyamines, depending upon the type of stress, the plant species and the time of stress application. [35] Polyamines have been proposed to participate in the metabolism, plasticity and possibly survival of higher plants subjected to environmental stress. [36] The effect of NaCl has been attributed to changes in osmotic potential resulting from reducing water content and specific toxic effects caused by accumulation of sodium and chloride ions. Salt stress may trigger polyamine accumulation independently of any osmotic component, even after short-term exposure. [37] Ali [38] observed that PA is related to regulatory effect in maintaining the structural and functional integrity of membranes in tomato.

Carrizo et al.[39] have reported that Cad was only present in transformed root culture of *Brugmansia candida;* where as other part of plants were devoid of Cad. On the other hand,

culture of cucumber cotyledon showed higher amount of Cad. [40] Cad content increased more during bud differentiation of callus than the root differentiation of callus, but that has been more remarkable than of non-differentiated callus. [32,41]

Low callus formation was observed in the explant treated with either with metal stress. Cad ameliorates the growth of the Callus under stress conditions (Fig. 4; 5) may be modifying the metabolites. Salinity and metal stress generally altered the processes of plant resulting in stunted growth. Since, stressed seedling growth alleviation in the presence of Cad is implicated here to enhance protein and biomass (Fig. 4, 5) to a great level; therefore, it was pertinent to determine the enzyme nitrate reductase (EC 1.6.6.1) in cultured cells as well. Callus showed sharp increase in NRA with Cad supplementation (Fig. 6) could be through increasing the enzyme protein either by elevating transcription or translation. Gulati and Jaiwal [42] also reported increase in NR activity in callus tissues of *Vigna radiata* differing in salt tolerance. Apparently, it may suggested that Cad potential to induce tolerance could be through elevating metabolites related with growth, required further experiment to know it more precisely.

The biosynthesis of Cad has been assumed to provide essential cellular diamines when Put biosynthesis via decarboxylation of ornithine is inhibited. [36,43] There are suggestions that plant regeneration in long-term rice cultures due to the accumulation of PA's could be restored through the modulation of PA metabolism. Cad is maintaining the stressed callus growth through maintaining the metabolites and ionic homeostasis. These findings may be useful for promoting and checking the potential of plant regeneration by Cad in many other cereals or certain recalcitrant species.

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