NBU Journal of
Plant Sciences

Vol. 3, March 2009
ISSN No. 0974 6927

Role of germination induced peptide pool in plant tissue culture

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Abstract
Low molecular weight peptides (ranges from 3.0 KDa to 0.5 KDa) were extracted and purified from rice, wheat, chickpea and mungbean through cryocrushing, cold centrifugation, ether fractionation, cation and anion-exchange column chromatographic separation, lyophilisation and ultrafiltration. The profile of heterogeneous peptides was detected through one dimensional paper chromatography. Peptide fractions isolated from different germination hours of mungbean enhanced mitotic index, more particularly prophase of root tip of *Allium cepa* L. and executed definite control over morphogenesis of excised and cotyledonary embryo culture of chick-pea seeds (*Cicer arietinum* L.). Restricted callogenesis in carrot pith culture was observed by the application of wheat peptides without any other hormones. Our speculation is that the peptides can mimic the action of hormone and behave as novel kind of bioactive molecule through which the physiological responses can be modulated.

Keywords: peptides, bio-activity, amylase induction, embryo culture, pith culture

The growth and development of higher plants can be considered to be characterized by the execution of cell division, expansion and differentiation along two axes: the apical-basal and radial patterning. In evolutionary terms, the apical-basal axis of development can be considered to have a strong selective advantage based upon plant competition for light, water and nutrients. Because plants are sessile organisms, their success in a particular environment will depend on their ability to integrate a complex range of external and internal information that may vary from time to time.

It is now fully recognized that plants exhibit greater morphological and developmental plasticity than animals. This conclusion has emerged as a result of integrating the data from molecular biological and genetic approaches with data gained from whole-plant physiological investigations (Trewavas and Knight, 1994). This organogenic plasticity in plants is coordinated by complex interplay between diverge signaling systems, leading to its immediate translation in the rate and plane of cell division, and cell expansion. The totipotency and plasticity of plant cells and tissues can be vividly exhibited when they are cultured *in vitro* with changing hormonal profiles. Basically plant tissue culture relies on the fact that more plant cells have the ability to regenerate a whole plant. Importantly totipotent cells must be able to differentiate not only into any cell in the organism, but also into extra-embryonic tissue associated with the organism. During development, the activities of plant hormones such as auxins, cytokinins, ethylenes, gibberellins and abscisic acid depend on cellular context and exhibit interactions that can be either synergistic or antagonistic. For example, auxin can suppress cytokinin biosynthesis (Nordstrom *et al.*, 2004), auxin and cytokinin can act synergistically to induce ethylene biosynthesis (Vogel *et al.*, 1998) and ethylene can modify auxin responses and meristem function (Souter *et al.*, 2004; Stepanova *et al.*, 2005).

To date, researchers have identified four major group of peptide-ligand-receptor pairs in plants (Ryan *et al.*, 2002), which are involved in a variety of developmental processes, such as wound responses, cellular dedifferentiation, meristem organization and self-incompatibility. However, these must only be part of the story, because plant genome sequencing has revealed many genes predicted to encode small peptide ligands and receptor-like kinases, whose function remain to be uncovered (Shiu and Bleecker, 2001). Furthermore, induced mutation in prohormone processing proteases has been shown to disrupt plant growth and development.

In this context peptide fractions isolated from different germination hours of *Vigna radiata* cv. Sonali B1 were analyzed for their bioactivity related to morphogenesis, cell division and dedifferentiation. Now, proper embryo development and germination are the learning phase for any plant to face the challenge of its adverse world. These two phenomena set the 'programming' of upcoming metabolic machinery, which will last till the death with minimum 'tuning'. 'Germination' is that crucial period where autotrophic plant also behaves heterotropically, which is an excellent manifestation of its selfishness and dependence, and slowly empowers its machinery for their future 'autotrophic' nature. This 'switch over' is controlled by a number of intrinsic factors, triggered by internal and external signal perceptions. This report is an attempt towards the understanding of changing pattern of peptide bioactivity isolated from different germination periods (i.e. – hrs of

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germination) of sonamung [*Vigna radiata* (Wilczek) cv. *sonali* B1] and also the interaction of these peptides with different hormones controlling growth and development.

Material and methods:

Plant Materials for Germination

Seeds of dicotyledonous plant material, mung bean, *Vigna radiata* (B1 *sonali* var.) were collected from Central Pulses Research Institute (C.P.R.I.), Berhampur, West Bengal, India. Seeds were weighed (each set of 250g.) and allowed to culture in sterile petri-plates with absorbent cotton supplied with modified Hoagland solution with one-half strength major nutrients and full-strength micronutrients. The nutrient solution was aerated continuously and renewed weekly. Experiments were conducted inside a controlled environment growth chamber with following conditions: 14-h light period with a light intensity of 350mmol m^{-2} s^{-1}, 25 °c / 20 °c day / night temperature, and 80% relative humidity. The plant materials were grown for different germination hours like 0h, 8h, 24h, 32h, 40h, 48h, 56h, 64h, 72h, 5days, 6days and 7days respectively and treated as different sets for definite experimental objective. Likewise certified seeds of rice, wheat and chickpea were also processed aseptically and cultured as stated above.

Isolation and Purification of Low Molecular Weight Peptides

Extraction: 100 g of seedlings were cut into pieces, rinsed with 0.2% sodium hypochloride for removing contamination and washed thoroughly with sterile distilled water. The seedling pieces were frozen in liquid nitrogen, crushed and extracted with chilled distilled water with a measured amount by blender at 4°C in cold room. The material was centrifuged at 10,000 rpm for 30 min using protease inhibiter PMSF at 4°C to precipitate the unwanted materials. The supernatant was collected and stored in deep freeze (-20 °C) for further study.

Ether wash: The extracts were subjected to ether wash at acidic pH (5.5) to remove endogenous hormonal impurities, fats, lipids and oils.

Ion exchange chromatography: The extracts were purified through cation and anion exchange resin (Dowex 50 and Dowex 1, Sigma Chemical Co., USA), filled in two-glass column (60 cm x 2.9 cm, 1.6 meq/ml.). Freshly prepared 3(N) ammonia and 1(N) HCl were used for elution of those peptides from the cation and anion exchanger column respectively. The ammonia and HCl were made free from extract solution through a liquid nitrogen trap fitted to a Lyophilizer (Lyolab BII). The whole extract was freeze dried to smaller volumes.

Ultrafiltration: The lyophilized material obtained from each set of respective germination hrs were separately undergone ultra-filtration through a millipore stirred cell fitted with 10,000 Da (YM10, Amicon), 3000 Da (YM3, Amicon) and 500 Da (YC05, Amicon) cut off filter paper separately and the filtrate between 3000 to 500 Da were collected. Precautions were taken for removal of amino acids from plant extract by using 500 Da cut off ultrafiltration for three times in each case. The ultrafillterd samples were lyophilized and dissolved in 10 ml distilled water (for each set) and stored in deep freeze (at -80 °C).

Paper Chromatography

100 ml (1 g fresh weight equivalent peptide) of each isolated peptide solution (peptide solutions of 8h, 24h, 32h, 40h, 48h, 56h, 64h, 72h, 5days and 6 days of germination) was loaded onto Whatman No-1 chromatography paper (size-46 cm x 57 cm, thickness-0.16 mm), and separated by descending chromatography with Isopropanol: Ammonia: Water(10:1:1 v/v).After removal of solvents the paper was sprayed with freshly prepared Ninhydrin location reagent (0.2g ninhydrin was dissolved in 100 ml methanol) (Plummer, 2005) on paper. The paper was placed in oven at 70°C for a very short period. When the bluish-purple spots were developed then it was taken out of the oven and documentation was done.

Cytogenetic Studies in Allium cepa root meristem cells

For determination of cytogenetic parameters, the experiments were carried out by the method of Konuk *et al.*, 2007. Roots of *Allium cepa* L. were treated with 10, 5 and 1 ppm peptide (lyophilized dry weight after ultrafiltration) solution isolated from three days old seedlings of rice, wheat, chickpea and mungbean. Since almost one day is required for completing the cell cycle of Allium cepa, experimentation periods were fixed for 24 h. After treatment, root tips were fixed in ethanol and glacial acetic acid mixture (3:1) of another 24 h at 5° C. Microscopic slides were prepared by squashing the tips in acetocarmine 1% (w/v). Mitotic phases were observed and photographed under Olympus research microscope and mitotic index were calculated for each dose introduced.

Plant Materials for Embryo and Pith Culture

Carrot Pith Culture Carrot taproots purchased from the local market were used as plant materials for the study. The carrots were chosen according to their tenderness, smoothness and freshness. After removing 5 cm sections from both ends, the carrots, were peeled and washed followed by immersion in 70 % ethanol for 20 min. Sterilization was carried out in 3% sodium hypochlorite solution for 15 min, to which two drops of Tween 20 were added. This was followed by rinsing three times in sterile distilled water. About 2 mm thick discs were cut from the carrots in such a way so as to include the xylem, phloem and the cambium. The cubes were inoculated onto the culture medium, maintaining polarity.

Excised and cotyledonary embryo culture of Cicer arietinum: Seeds of Kabuli type chickpea (*Cicer arietinum* L.) were surface sterilized as above, seed coats were removed aseptically and cotyledonary embryo or excised embryo proper were used for tissue culture.

Culture Medium and Culture Conditions

The culture medium used for all experiments was based on MS medium (Murashige and Skoog, 1962) to which

0.8% Difco Bacto Agar and different concentration of peptides were added, but no plant growth regulators. The control consisted of basal MS medium supplemented with 2, 4-D and BAP. Medium pH was adjusted to 5.8 with KOH before adding the agar. All media were sterilized by autoclaving at 121° C and 101 KPa for 20 min. Cultures were incubated at 24 ± 2° C and exposed for 16 h per day to an illumination of 16 $\mu molm^{-2}s^{-1}$ provided by white fluorescent lamps.

Determination of Peroxidase and Total Protein

Crude Peroxidase extracts were prepared by grinding a fresh tissue in 0.1 M phosphate buffer (pH 7.0). The homogenate was centrifuged at 15,000g for 15 min. The total Guaiacol Peroxidase activity of supernatant was determined spectrophotometrically at 470 nm (Krsnik-Rasol, 1991). Enzyme activity is expressed as A_{470} (g. fresh weight)$^{-1}$min^{-1}.

The total protein in the supernatant was determined by the method of Lowry *et al.* (1951) using Bovine Serum Albumin Fraction V as standard.

Results and Discussion

In spite of the importance attained by plant tissue culture and of many studies that have been conducted on related developmental process, there are still many aspects of growth and dedifferentiation that are not fully understood. Recent progress achieved on understanding the interaction between exogenously added plant growth regulators over the concentration of endogenous hormones, together with the involvement of sensitivity of the tissues to particular hormone groups, might help clarifying the occurrence of divergent patterns in callus induction and somatic embryogenesis (Jimenez 2005). In the present investigation, callus induction in chickpea was observed onto MS media containing different concentrations and combination of 2,4-D, NAA, IAA, BAP and Kinetin within 20 days of incubation of cotyledonary embryo explants depending upon the concentration and combination of hormones. Callus induction was noticed in all media formulations. But there was a wide range of variation in percentage of callus formation and average fresh weight of callus. The highest percentage of callus induction (90%) was observed on MS media containing 3.0 mg/l 2, 4-D and 1 mg/l BAP. The results are in concordance with Huda *et al.* (2003) where they showed that 2, 4-D and BAP were the most ideal hormonal combination in MS medium for induction of callogenesis in *Cicer arietinum* L. Highest callus growth in terms of fresh weight [0.576 g] was observed in MS medium fortified with 2 mg/l 2,4-D, 0.5 mg/l BAP and 100 µg/ml of rice peptides. Colour of the calli was mostly light brown to whitish green and light green. It was observed that only light green calli produced shoot buds. From this study it was observed that 2,4-D without cytokinin could induce callus but for better proliferation auxin (2,4-D, NAA and IAA) and cytokinin were required. Maximum shoot buds were proliferated from MS media containing 3 mg/l 2,4-D + 2 mg/l BAP. The shoot buds first appeared as nodular growth from cotyledonary axils after 22 days of culture and at the end of one month these nodules increased in size and produced leaf primordia.

No remarkable enhancement of callogenesis was observed from our experiments when the peptides of rice, wheat, mung and chickpea were incorporated in both auxin and cytokinin fortified medium. But percentage of callus induction was strikingly lower when only auxin or cytokinin is present in the medium. In this condition, if the above mentioned peptides were combined with either of these two hormones (2, 4-D or BAP) prominent increase of callus tissue was noticed and peptides isolated from one week old *Vigna radiata* was most responsive. As the bioactivity of *Vigna radiata* peptides were observed to be most prominent among the four, only *Vigna radiata* peptides isolated from different germination hours of growing seedlings were taken for further bioassays. Induction of callus gradually

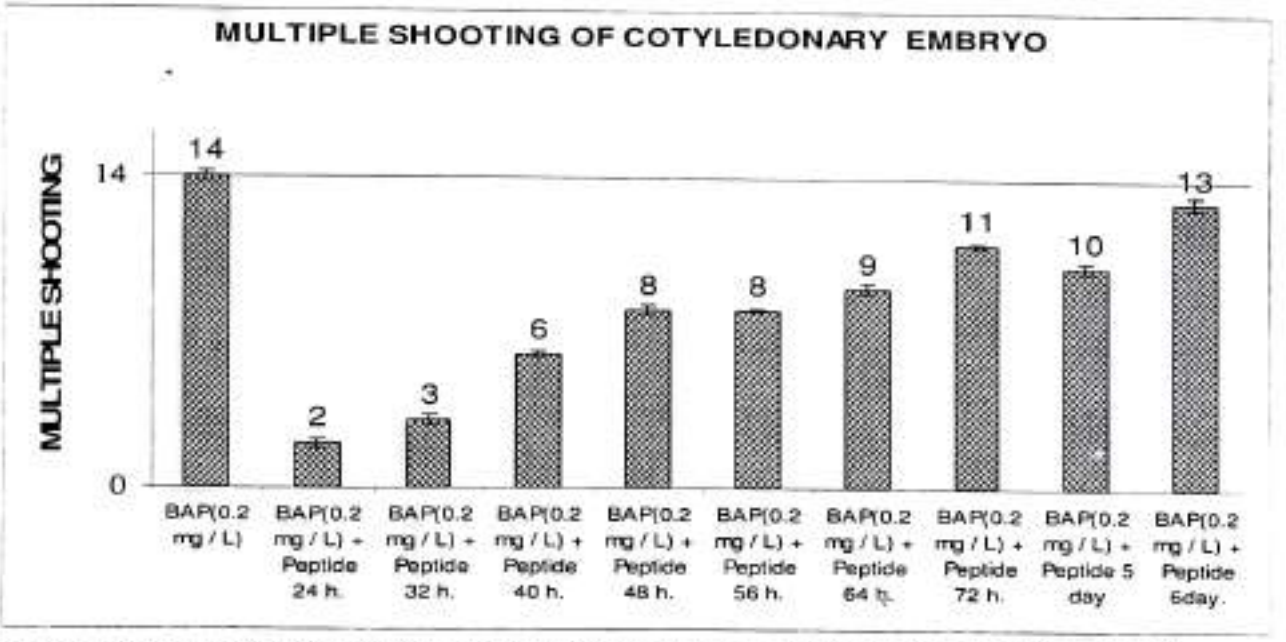

Fig.1: Multiple shooting of cotyledonary embryo of *Cicer arietinum* L. after application of peptides isolated from germinating mung bean seedlings

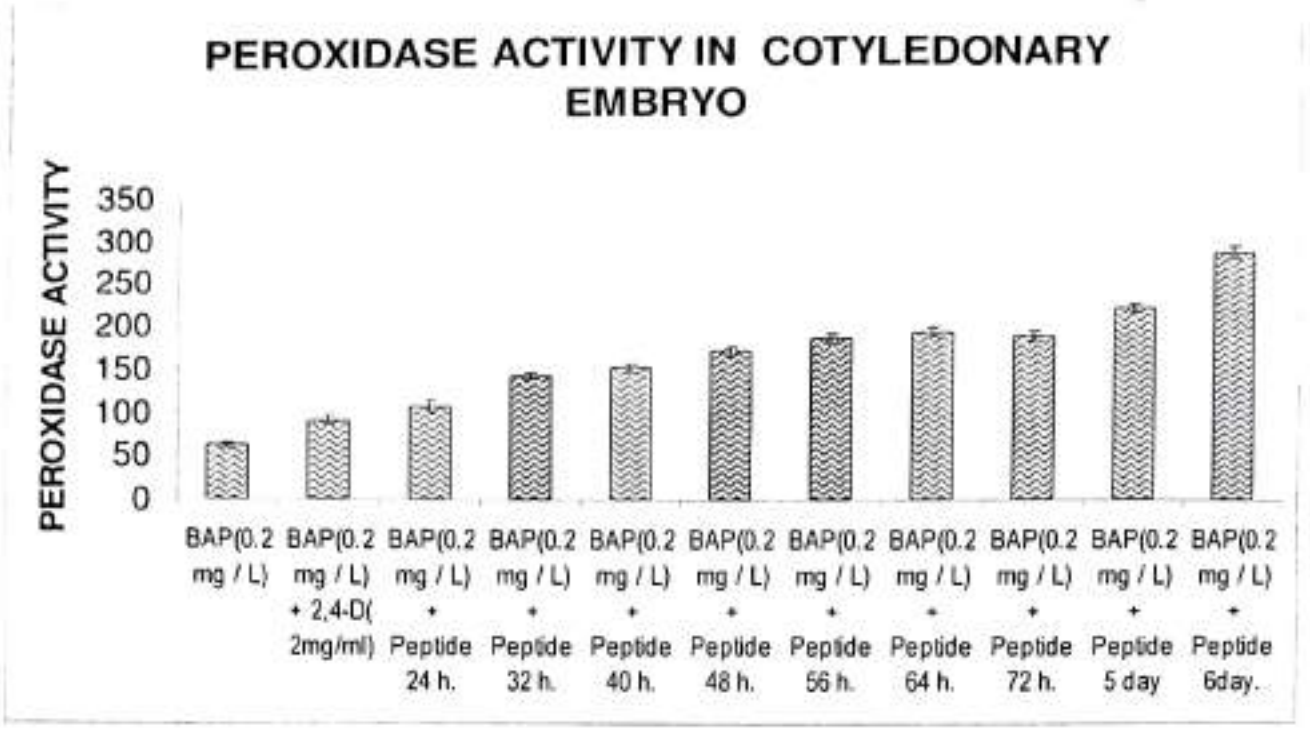

Fig. 2: Peroxidase activity of cotyledonary embryo of *Cicer arietinum* L. after application of same peptides

increased in MS media fortified with 3 mg/l of 2,4-D and 100 µg/ml of peptides isolated from 8 to 64 hours and the maximum response was obtained at 2,4-D + 64 h peptides where callogenesis was observed in 86% of the explants. The minimum response of 58% callogenesis was observed at 2, 4-D + 5 days peptides combination whereas the callus induction was only 49% when only 2, 4-D alone was present in MS medium. So the results of the present study conclusively demonstrated that the induction of callogenesis was amplified significantly when peptides of germinating mung bean were associated with auxin. Restricted callogenesis was observed at excised cotyledon nodes when the same peptides were combined with BAP (Fig. 4 xi A & B). In BAP combined peptide medium, the calli were fragile, light green and embryogenic but no callogenesis was documented when BAP was only present. Peptides in BAP combining medium enhanced the potency of regeneration of multiple shoots (Fig.4 i-x) and maximum percentage of multiple shooting was observed at 1 mg/l BAP + 72 h peptides with almost 79% regeneration capacity (Fig. 1). Maximum multiple shooting was only 53% when BAP alone was treated with explants. So in true sense germination induced peptides improved the bio-efficacy of both the classical hormone 2,4-D and BAP. But peptide alone has no capacity to induce and proliferate callogenesis and multiple shooting in *Cicer arietinum*. Also it was further documented that BAP (1 mg/l) combined peptide (100 µg/ml.) medium reduced the number of multiple shoots per plant, though the same combinations enhanced the percentage of regeneration of multiple shoots from cotyledonary explants when compared with BAP (1 mg/l) alone.

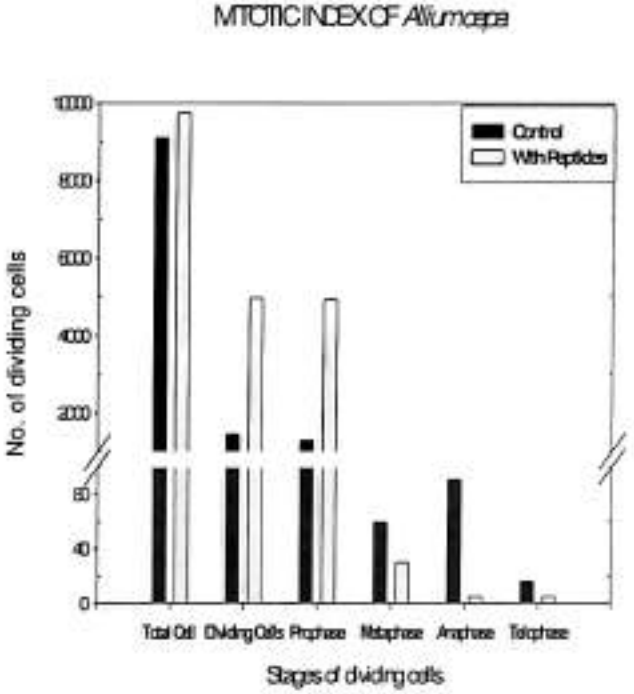

Fig. 3: Mitotic index of *Allium cepa* L. root tip after application of 1 ppm peptides isolated from 3 days old mung bean seedlings

Legumes generally and chickpea particularly is supposed to be recalcitrant and difficult to manipulate with *in vitro* cultures. The alteration in morphogenesis that was achieved due to application of plant peptides with either auxin or cytokinin may be considered significant because it was required to overcome the recalcitrant behaviour of chickpea. Though in chickpea callogenesis was not accomplished only by peptides due to this obstacle, in carrot disc culture (Fig. 4 xiii) restricted callogenesis was observed in lower frequency (16.82%) by the application of wheat peptide alone in

MS medium without the combination of any other hormones. Generally in carrot optimum callogenesis was observed in combination with 3 mg/l of 2, 4-D and 1 mg/l of BAP (Fig. 4 xii), as established from our experimental results and the findings were also corroborated with the views of other developmental biologists (Jimenez *et al.*, 2001). No callus induction has been documented in carrot root disc culture in MS without any hormones or peptides.

Form the above discussion it may be concluded that isolated peptides somehow mimicked or influenced the callus inducing patterns of chickpea or carrot, regulated by auxin. The results were again reasonably fascinating when the peptide fractions of different germination hours [0 h. to 6 days] were subjected for incubation with mitotically dividing meristematic zones of *Allium cepa* for observing their direct effect on cell division. Like callus induction mitotic index of *Allium cepa* root tip was also increased with the application of *Vigna* peptides isolated from germinating and post-germinating phases of 0 h to 7 days of seedling age. Mitotic index was maximized with peptides representing 64 h of seedling age and after 3 days; the effect of isolated peptides was gradually retarded. When the bioactivities of 64 h peptides were further elucidated with paper chromatographic separations (Fig. 4 xiv), it was observed that the mitotic index drastically increased with two fractions (R_f zone 1 and R_f zone 3) but the total improvement was the reflection of enhancement of prophase. This is again fairly interesting because it shows that a preliminary signal induced the mitotically active cells to enter in dividing phase (M phase) but due to lack of some components, most of them were specifically arrested in prophase (Fig 3). In contrast, application of peptides separated from R_f zone 4 reduced the whole mitotic index of the cells.

Cell cycle is a critical and disciplined phenomenon, which generally shows no by-passes towards the decisive result. The basic machinery of cell cycle is highly conserved. In particular, many cellular events during this cycle progression are controlled by cyclin-dependent kinases (CDKs). Plants possess a unique class of CDKs (B-type CDKs) with preferential protein accumulation at G2/M-phases and control the entry into mitosis from G2 of interphase (Fobert *et al.*, 1996, Segers *et al.*, 1996). The regulations of cell cycle control of animals are subsequently different in the embryonic and somatic cells (Inzé and De Veylder, 2006). Sauter *et al.* (1998) hypothesized that zygotic regulation of the first cell cycle may contribute to a greater adaptive ability of plants during early embryogenesis, unlike animal embryos in which the cell division is more rigidly fixed before fertilization. This analysis also might lead to the prediction that embryonic tissues of plants are more totipotent than animals with greater morphogenic plasticity which might be due to the contribution of diffusible peptides in addition to classical hormones. Endogenous bioactive peptides, *i.e.* plant-derived peptides that evoke specific cellular responses, provide the most direct evidence for a general role of peptides in the regulation of plant growth

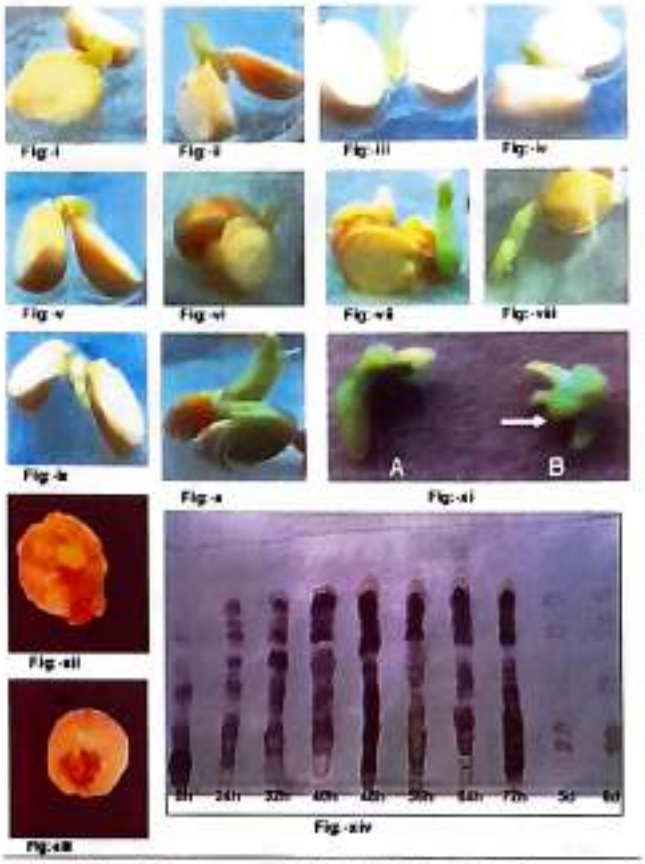

Fig. 4: i = Treatment with BAP; Fig:- ii = Treatment with BAP + 24 h Pep.; Fig:- iii =Treatment with BAP+ 32 h Pep.; Fig:- iv = Treatment with BAP+40 h Pep.; Fig:- v = Treatment with BAP+48 h Pep.; Fig:- vi = Treatment with BAP+56 h Pep; Fig:- vii = Treatment with BAP+64 h Pep.; Fig:-viii = Treatment with BAP+72 h Pep. Fig:-ix =Treatment with BAP+5 day Pep.; Fig:- x = Treatment with BAP+ 6 day Pep.; Fig:- xi A=Normal culture; Fig:- xi B=Treatment with BAP+ 6 day Pep.; Fig:-xii =Normal carrot pith culture; Fig:- xiii =Culture with 6 day Pep; Fig; xiv = One Dimension Paper Chromatography of germinating mung bean

and development. Already the peptides like POLARIS, Phytosulfokine, ENOD 40, Rapid Alkalinization Factor (RALF) and Clavata 3 (CLV3) have been shown to have pivotal roles in plant growth and development (Matsubayashi and Sakagami, 2006). On the other hand, different physiological upsets, like wound or pathogen (e.g. *Agrobacterium tumifaciens*) invasion, can stimulate mitotic division.

As these peptides of ultra low molecular weight range were functional from extracellular medium and showed a direct effect on mitotic index, they must have diffusible properties, which were thought to be much better suited to penetrate the rigid cell walls between adjacent cells as compared to large peptide hormones. Research in recent years, however, has indicated that peptides may be widely used as chemical signals in plants as well. Like Phytosulfokine these peptides led the cells towards the entry into mitotic division phases but unlikely unable to complete the rest. One thing we must take in care that the source of peptides is mainly developing embryo and the test material is somatic tissue. Therefore, it is quite logical to say that these

peptides promoted the cells towards the divisional phase by creating immense impact over cell division in developing embryo. As a matter of fact, the peptides of R_4 zone-4 have negative regulation on both cell division and amylase synthesis. So, it is reasonably justified to assume that living system is so disciplined and balanced that it always maintains a feedback inhibition within them. Though it is somewhat surprising for the same molecule to inhibit both of the above actions but not impossible. Our investigations with germinating sonamung support the above view.

Like all developmental processes, callogenesis and organogenesis was also associated with alteration of oxidative stress. Peroxidase activity as well as thiol and ascorbic acid contents can be considered as important metabolic markers of oxidative stress during development of somatic embryo (Norgaard, 1997). When the peptides were tested for regeneration capacity and callogenesis in *Cicer* tissue, it was observed that peroxidase activity increased (Fig 2) considerably in the callus at the end of the culture. But the highest peroxidase activity was determined in the embryogenic line at 6 days of culture in the presence of growth regulator BAP and peptides at a concentration of 100 µg / ml. The minimum peroxidase activity was estimated in habituated tissue grew in presence of 2,4-D and BAP and formed white swollen aggregates of shoots and hypocotyls with strongly reduced potential for somatic embryogenesis. An increase in peroxidase activity was also recorded in those flasks, where tissue became looser and after few days embryoids were noticed. After formation of globular to heart shaped embryoids, peroxidase activity was dropped 3 to 4 times, as compared with callus tissues from which they originated. It was also detected that peroxidase activity was enhanced during multiple shooting formations in cytokinin peptide combined medium. When the tissue specific activity of peroxidase was studied, it was noticed that the peroxidase activity was about three times higher in bushy multiple shoots than that of single leaf or shoot. This is in agreement with Krsnik-Rasol (1991) where they claimed that bushy transformants and crown gall tissue extracts were 6 to 7 times higher level of enzyme activity than in normal leaves.

As a conclusion, these results shed light on the bioactivity of peptides which play a significant role in callogenesis and morphogenic alteration. These data show that Peroxidase activity can also be associated with organogenesis under the influence of peptides of ultra low range. Further investigations will be required to establish the molecular mechanisms of regulation of peptides controlling morphogenesis and cell division.

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