CHAPTER-X

EFFECT OF SEED COAT PHENOLICS ON GERMINATION AND GROWTH PHYSIOLOGY AND THEIR INTERACTION WITH ISOLATED PEPTIDES

10.1 INTRODUCTION

It is well known fact that mung or green grams [*Vigna radiata* Wilczek.] have been consumed by humans since the earliest practice of agriculture (Phillips and Mcwaters, 1991) and they have various food uses in Asia and Africa. One of the major limiting factor of utilization of this mung bean as food is the presence of antinutritional factors such as trypsin inhibitors, oligosaccharides and phenolic compounds (Chavan *et al.*, 1989). Phenolic compounds (tannins in particular) are important group of such antinutritional factors. They are able to form complexes with food nutrients such as minerals and proteins, thus rendering them less soluble or less susceptible to enzymatic degradation and less available for absorption (Towo *et al*, 2003). Legume seeds also constitute one of the cheapest and richest sources of protein but its protein quality is low due to presence of these tannins and free phenolic acids.

However phenolic compounds have a beneficial role as well. They are naturally concentrated in the seed coat (Preet and Punia, 2000) where they play a major role in the physical and chemical defense system of the seeds when exposed to environmental factors such as oxidative damage and microbial infections thus contributing to antioxidant and antimicrobial activity (Trozynska *et al.*, 2002). Physiologically phenolic compounds affect plant growth and germination in several cases. A series of experiments in both field and laboratory have indicated during the last two decades a role for a number of phenolic derivatives as allelopathic agents. These are chemicals exerted by the plants, which may be autotoxic or affect the growth of other plants in the environment (Rice, 1984). It has also been found that flavonoids, and especially tannins, have a role as feeding deterrents, and protecting plants from overgrazing by many animal species (Swain, 1977).

It has been suggested that phenolic acids from legume green manures may contribute to weed control through allelopathy. For this, oxidation reactions of phenolic acids in soil and the subsequent effects of oxidation upon phytotoxicity were investigated. Soils were reacted for 18 hours with 0.25 mmol/L benzoic and cinnamic acid derivative solutions and manganese release from the suspension was used as a marker for phenolic acid oxidation. The extent of oxidation in soil suspensions was in order of 3,4-dihydroxy->4-hydroxy-3-methoxy->4-hydroxy->2-hydroxy- substituted benzoic and cinnamic acids. The same ranking was observed for Voltametric peak currents of the cinnamic acid derivatives. This suggests that the oxidation of phenolic

acids is controlled by the electron transfer step from the sorbed phenolic acid to the metal oxide. A bioassay experiment showed that the 4-hydroxy, 4-hydroxy-3-methoxy and 3, 4-dihydroxy-substituted cinnamic acids were bioactive at 0.25 mM/L concentration. Reaction with soil for 18 hours resulted in the elimination of bioactivity of these three cinnamic acids at the 5% significance level. The oxidative reactivity of phenolic acids may limit the potential of allelopathy as a component of an integrated weed management system. However, the initial phytotoxicity after soil incorporation may coincide with the early, critical stage of weed emergence and establishment, so that the allelopathic phenolic acids may still play a role in weed management despite their reactivity in soil systems.

Beside these, phenolic acids play critical role in preventing germination. During the development and ripening of rye caryopses, a gradual increase in precocious germination ability of the grain was observed. The enforced dehydration stimulated the process of precocious germination of developing and ripening rye caryopses. Phenolic acids, present in them, were found to be responsible in preventing precocious germination and acclimation to dehydration of developing and ripening rye grains. Five phenolic acids were analysed in rye caryopsis (*i.e.* vanillic, caffeic, ρ-coumaric, ferulic and sinapic Acid) by high pressure liquid chromatography, three of which (ρ-coumaric, ferulic and sinapic) were found as free phenolic acids in rye caryopsis at the beginning of development. During dehydration, the total level of free phenolic acids in rye caryopsis decreased, although much increase of sinapic acid content was observed after dehydration treatment in all investigated samples of caryopsis of various ripeness. Weidner and Paprocka (1997) proposed that dormancy of cereal caryopsis might be at least partially controlled by the high level of free phenolic acids through their inhibitory effect on germination and cell division.

Because plants are non motile, the choice between continued dormancy and germination in the seed is of critical importance to plant survival. The establishment of seed dormancy in higher plants is influenced by environment cues such as moisture, light and temperature. Seed dormancy has been defined as the failure of an intact, viable seed to complete germination under favourable conditions (Bewley, 1997). Since in *Arabidopsis*, removal of the seed coat allows germination of non-germinating and strongly dormant genotypes, *Arabidopsis* should be described as coat-enhanced dormancy (Bewley, 1997). Seed coat imposed dormancy in particular is part of the seed survival strategy of many species (Werker, 1981; Kelly *et al.*,

1992). The seed coat exerts its germination restrictive action most of the time by being imperbiable to water and/or O_2 by its mechanical resistance to radicle protrusion. These properties have been positively correlated with seed coat colour due to phenolic compounds in diverge species *e.g.* Red seeds of Charlock (*Sinapis arvensis* L.) exhibit a reduced dormancy compared with black seeds (Duran and Retamal, 1989). In legumes, white seeds imbibe more rapidly and also suffer greater imbibition damage, which affects their viability (Wyatt 1977; Werker *et al.* 1979; Powell 1989; Kantar *et al.* 1996) than coloured seeds and then germinate earlier. In wheat, the strongest dormancy is associated with the red seed coat colour, whereas the lines with white seed coats are non-dormant or weakly dormant (Gfeller and Svejda, 1960; Mares, 1994). Dark seeds of prosomillet (*Panicum miliaceum* L.) have earlier seed coats, imbibe and germinate more slowly, suffer less imbibition damage and therefore persist longer in soil than light coloured seeds (Khan *et al.* 1996). It was hypothesised that during dehydration of seeds, an enzymatic oxidation of phenolic compounds in presence of O_2 might render the seed coat impermeable to water (Marbach and Mayer, 1974; 1975). In *Arabidopsis*, phenolic compounds and their derivatives present in the inner layer of the testa, called endothelium, affect seed coat properties that influence germination. The flavonoid polymers may play a major role in limiting not only water entry, as seen in legumes, but also oxygen supply to the embryo, as reported by Corbineau and Come (1993) for cereals, and by contributing to the mechanical resistance of the testa. They may also inhibit the leaching of germination inhibitors out of the seed, as proposed for charlock (Edwards, 1968; 1969). Seed dormancy in *Arabidopsis* can be overcome by, or seed germination is activated by the common germination factors such as after-ripening, light, cold treatment (also called stratification) and chemicals such as gibberellins and KNO_3 (Derks and Karssen, 1993). The plant hormone gibberellin plays an important role in promoting seed germination without exogenous GAs (Koornneef and van der Veen, 1980) *e.g.* in plants such as *Arabidopsis* and tomato (*Lycopersicon esculentum*). GA can also play a role in controlling the abundance of β-glucosidase, which might be involved in the embryo cell wall loosening needed for cell elongation and radicle extension. Many studies have focused on the role GAs in dormancy breakage (Metzger 1983; Hilhorst and Karssen 1988; Derkx and Karssen 1993) and in the mobilization of seed reserves during seedling establishment. In the early events occurring during seed germination, two main mechanisms have been documented.

The first one includes in the induction of endosperm and seed coat weakening. In the second mechanism, GAs would be involved in resumption of cell cycle activity during germination, as documented for example in tomato seeds (Liu *et al.* 1994). To investigate the role of GAs in germination of *Arabidopsis* seeds, the proteome of GA-deficient *Arabidopsis* in which GA deficiency is conferred were characterized by the *ga1* mutation (*ga1* allele). The *GA1* gene codes for the enzyme *ent*-copalyl di phosphate synthase that catalyses the first step in the GA biosynthetic pathway (Sun and Kamiya, 1994). In addition, the effect of PAC on the proteome of wild type (WT) seeds were also analysed during germination. *Arabidopsis* seed germination is highly sensitive to this compound (Debeaujon and Koornneef, 2000), which blocks GA biosynthesis and thereby radicle emergence through inhibition of the enzyme *ent*-kaurene oxidase (encoded by the gene *GA3*). It was reported that radicle protrusion was strictly dependent on exogenous GAs (Koornneeff and Van der Veen, 1980; Karrssen and Lacka, 1986; Debeaujon and Koornneef, 2000). A comparison of the proteome from WT and *ga1* dry mature seeds revealed six polypeptides that showed a substantially higher accumulation level in the *ga1* seeds than in the WT seeds. They were all identified as 12s globulin precursors by MALDI-TOF analysis. Thus, it has been suggested that GAs play a role in the accumulation of seed storage proteins during maturation, presumably by specifically impending the accumulation of 12s globulin precursors. The processing of these precursor forms affect the accumulation of 12s globulins within protein bodies because unprocessed forms can only associate as trimers, whereas mature globulins associate as hexamers. 11-12 s globulins are abundant seed storage proteins, which are widely distributed among the higher plants. They are synthesised during seed maturation on the mother plant in a precursor form consisting of a single protein chain of about 60 KDa. Later, the precursor form is cleaved, yielding the mature globulins which are generally found in storage protein bodies of dry mature seeds. These are composed of six subunit pair that interacts covalently. Each of these pairs consists of an acidic ά-subunit of Mr 40,000 and a basic β-subunit of Mr 20,000, covalently joined with each other by a single disulfide group. These subunits are subsequently broken down during germination and used by germinating seedling as an initial food source (Bewley and Black, 1994). However, it has been noted that the *ga1* mutant plants experienced the action of exogenous GAs during their growth; these plants were sprayed once a week with 10 mM GA_{4+7} to stimulate elongation, growth and anther development. This suggests that the action of

exogenous GAs did not completely mimic that of endogenous GAs in terms of concentrations and / or spatial and temporal patterns of expressions. The presence of mature 12s storage proteins in them, suggest that residual exogenous GAs were sufficient to induce the cleavage of the precursor molecules. For the mobilization of food reserves during germination and early seedling growth, the activity of lipid and carbohydrate degrading enzymes such as malate synthase (MLS) and iso-citrate lyase (ICL) is required, which are the unique to the glyoxylate cycle. Marriott and Northcote (1975) showed that the GAs stimulate the induction of isocitrate lyase activity during germination. *Arabidopsis* mutants, *icl-1* and *icl-2*, which lack the glyoxylate cycle because of the absence of key enzyme isocytrate lyase, demonstrated that the glyoxylate cycle is not essential for germination, but is important for seedling establishment and survival. Moreover, it is evident that resumption of cell cycle activity is a specific feature of early germination. Gallardo *et al.* (2001) revealed an accumulation of five proteins associated with cell cycle events during germination of the WT *Arabidopsis* seeds. These proteins were actin 7, α-2,4 tubulin, α-3,5 tubulin, β-tubulin and a WD-40 repeat protein. Tubulins are associated with cell division and cell enlargement. During cell division, they play an important role in separation of the organelles and daughter chromosomes (mitosis). Liu et al. (1994) demonstrated a GA-requirement for resumption of cell cycle activity during germination of tomato seeds. Cortical microtubules are formed in the radicle prior to protrusion with germinating tomato and cucumber (*Cucumis sativus*). These cortical microtubules are most likely associated with preparation of cell elongation. Out of five above-mentioned proteins, only α-2,4 tubulin showed a distinct pattern of accumulation when comparing the *ga1* mutant and the *WT Arabidopsis* seeds after 1 day of imbibition in water. This protein strongly accumulated in the *WT* seeds, and not in the *ga1* mutant seeds. Moreover, an accumulation of this protein occurred during 1 day of imbibition of the *ga1* seeds in GA_{4+7} solutions. Therefore, all these data support the conclusion that GAs control the accumulation of α-2,4 tubulin during germination. Furthermore, Huang and Lloyd (1999) showed that GAs stimulate α-tubulin acetylation and stabilize microtubules in maize suspension cells.

Phenolic compounds that are present in the testa interfere with the physiology of seed dormancy and germination. Recessive *Arabidopsis* mutant with pale brown seeds, transparent testa 12 (*tt12*), were isolated from a reduced seed dormancy screen. Microscopic analysis of *tt12* developing and mature testas revealed a strong reduction

of proanthocyanidine deposition in vacuoles of endothelial cells. *TT12* gene is expressed specifically in ovules and developing seeds. The phenotype of mutant and then nature of the gene suggest that *TT12* may control the vacuolar sequestration of flavonoids in the seed coat endothelium. As a consequence of their action on testa hardening, these compounds play an indirect restrictive role in seed germination by hampering radicle protrusion from the integuments and therefore are important determinants of seed coat-imposed dormancy (Debeaujon and Koornneef, 2000). In general, phenolics have the property of altering mitochondria and chloroplast membranes, hindering of energy transfer necessary to ion transport, as observed in spinach (Moreland and Novitzky, 1987). Coumarins seem to inhibit mitosis like colchicines, showing anti-microtubule effects (Corman, 1946). For phenolic acids, polyphenols (but not monophenols) seem to increase IAA-mediated growth by inhibiting IAA oxidative decorboxylation. This can be extended for flavonoids.

Every phenotypic expression, in a plant, is a direct and /or indirect reflection of its internal metabolism and the whole metabolism profile, starting from its germination, is nothing but a continuous interaction of diverse potential biomolecules. As like as other natural phenomena, every "metabolism" (though all of them, as a whole, are interconnected) has some inducing and inhibiting factors and mainly proteins, peptides, phenolics, phytohormones play those roles. Now, as each "control" is multi-factorial and each factor has multidimensional activity; so it is quite natural for a factor to act as inducer in one system and inhibitor in other. Ultimately the tussle between inducer and inhibitor results in fulfilment of any phenomenon.

Activity of protein in all stages of life is a well-established concept. Recently, a close neighbour of protein, i.e. peptide, has also established itself as indispensable, potential biomolecule in plant system. They mainly work as extracellular signals and have their wide spectrum effect on growth, development and defence. Talking about these peptides is always something different because they are small with very low molecular weight (less than 10 KDa.) and even their genesis can be – through proper genetic machinery, or catalytic effect of enzymes or even they can be of self replicating in nature.

In this chapter, the specific role of phenolics and peptides during germination and post germination phases were discussed. Here the basic aim is only to investigate the interactive and multi-coordinated pattern associated with the physiological processes of germination. And for this, I have taken Sona Mung (*Vigna radaita* B1 Sonali

Variety), for the extraction of low molecular weight peptides (3 KDa to 0.5 KDa.) and phenolics. Pure Gibberelic Acid and different synthetic Phenolic Acids were also used for this experiment. Wheat seeds (*Triticum aestivum* cv. Sonalika), Barley seeds (*Hordeum vulgare* Dwarf variety) and Sona Mung seeds were taken as test material. The overall report is designed with TLC, Capillary Electrophoresis of isolated peptides & phenolics from Sona Mung and bioactivity investigations through – Amylase Induction Test, Germination Percentage Test, Radicle Emergence Test and effect on Plant Height. For determining the interaction pattern of phenolic compounds and peptides, we combine phenolic compounds with wheat, rice, gram and mung peptides and performed the same α-amylase induction assay taking standard as GA (amylase inducer) and ABA (amylase induction inhibitor). As it is expected germination, radicle emergence and amylase induction were inhibited by the application of extracted phenolic acids from sonamung seeds. Not only those, peptides isolated from seedlings of different cereals and pulses which can able to induce amylase, when combined with these phenolic acids inhibited germination as well as amylase induction. So this study points on the phenolic acids as negative regulator of amylase induction signal and probably when combine with peptides, interfere with the bioactivity of peptides.

10.2 MATERIALS AND METHODS

10.2.1 Plant culture

Vigna radiata cv. Sonali plant culture; for this job, was done by following the methods and specific conditions as described in earlier chapters.

10.2.2 Isolation and purification of low molecular weight peptides

Isolation and purification of low molecular weight peptides (3k Da to 0.5 k Da) was performed as described earlier in Chapter-III Section 3.2.2.

10.2.3 Isolation and purification of phenolic compound

Fresh and dry 500 g mung bean seeds were crushed in a mixer and grinder with methanol and then heated on hot plate in order to concentrate it. It was then kept overnight to one day in freezer at 8°C and filtered. The filtrate was again concentrated to about 50ml and then, diethyl ether solution was added (450 -500 ml), shaken well in separating funnel. Ether part was taken and concentrated. After that, 5%$NaHCO_3$

was added, shaken well and bicarbonate part was taken. The residue (i.e- ether part) was shaken with 5%NaOH and NaOH part was taken out. Both bi-carbonate and NaOH part were reduced to pH 5 by adding dilute HCl. Then ethyl acetate was added and shaken well. The resultant ethyl acetate part was collected, removed and reconstituted in methanol, and then in chloroform solution. Phenolic compounds were separated from fractional crystallization of phenol chloroform immiscible solvent.

10.2.4 Capillary electrophoresis of phenolics

The extracted phenolics were subjected to capillary electrophoresis, Beckman P/ACE system 5010. Amount of sample loading in each case is 60µl with 50 second injection time. Hydrodynamic injection was implemented by applying a pressure of 20 psi to the sample vial. Detection wavelength of 214 nm, used neutral gas nitrogen, capillary volume of 50µm x 47 cm (neutrally coated), voltage-18KV (8.4 µamps, temperature-20°c) and detection time of 5 seconds was monitored. eCAP^{TM} citrate buffer pH-3 (20µM citrate) was used as running buffer. Identification was performed by synchronizing authentic marker peaks using standard phenolics: *o*-hydroxy coumeric acid (Mw.), protocatechuic acid (Mw.), gallic acid (Mw.), chlorogenic acid(Mw.), *o*-hydroxy cinnamic acid(Mw.), caffeic acid(Mw.), *p*-hydroxy benzoic acid(Mw.) and 4-hydroxy cinnamic acid (Mw.). All the chemicals used for this experiment were of analytical grade and purchased from Sigma Chemical Company, USA.

10.2.5 Amylase release test

Wheat (*Triticum aestivum* cv. sonalika) half seeds without embryo were taken aseptically and those half seeds were incubated separately with different concentrations of GA_3 solutions for 48 hrs under dark. Reducing sugar produced was estimated by 3, 5-dinitrosalicylic acid method (Nicholls and Paleg, 1963).

10.2.6 Germination percentage (g %) test

Seeds of sonamung [*Vigna radiata* cv. sonali] were incubated with different combination of test solutions separately for 24 hrs and after that the germination percentage was properly calculated.

10.2.7 Radical emergence test

After 24hrs of incubation of seeds of sonamung under different combination of test solution, the sizes of emerged radical were calculated.

10.2.8 Effect on plant height

Growth bioassay was done in terms of measuring plant height. For this, germinating barley (dwarf variety) [*Hordeum vulgare*] seeds were incubated with different combination of GA and extracted peptides for 3 days.

10.2.9 *In vitro* study for determining any possible interactions between peptides and GA

100μL of 10^{-4}(M) GA & peptide solutions at its bioactive concentrations were incubated aseptically at 10°C - 12°C for 24hrs. The peptides and Gibberellic acid were then spotted in chromatography paper and one dimensional separation was done with n-Butanol: Acetic acid: water. Then peptide spots were detected through Ninhydrin location reagent and GA spot was detected with 70%H_2SO_4 under short length ultra violet light.

10.3 RESULTS AND DISCUSSION

10.3.1 Profile of phenolic acids in the seeds of *Vigna radiata*

Considerable amount of phenolic compounds has been found to be present in *Vigna radiata* as determined by Phenol Reagent. The Folin-Ciocalteu method quantifies the total amount of phenolic hydroxyl groups present in the sample being assayed (Waterman & Mole, 1994) based on the ability of the phenolic hydroxyl groups to reduce the phosphotungstic-phosphomolybdate complex in the Phenol (Folin-Ciocalteu) reagent. In case of mung bean green (green gram *Vigna radiata* Wilczek.) and golden yellow (golden gram Vigna radiata Wilczek. cv Sonali) variety, the total phenol content is 6.43±0.80 & 4.02±0.60 mg catechin equivalent per gram seed sample respectively. The wide variation of phenolic content in Leguminous seeds has been observed elsewhere (2.23). Preet and Punia (2000) reported total phenolics in cowpea ranging between 7.79 to 9.35 mg catechin equivalent / gm. Towo *et al.* documented total phenolics ranging from 3.47 to 9.14, catechols from 1.58 to 3.51 and resorcinols from 1.41 to 5.37 mg catechin equivalent / g sample. Structural

grouping of phenolic resorcinols, catechols and galloyls is less reported and most authors emphasized that catechol and resorcinol contents were relatively higher than galloyls in the analyzing legumes. According to Barroga *et al.* (1985) polyphenols of mung bean has low protein precipitating capacity, relatively high flavonol levels and were concentrated in the seed coat. Soaking of seeds in water reduce the accessible polyphenol contents up to 50%. The high phenol content in the seed coat is because phenolic compounds in legumes are known to be concentrated in the seed coat (Preet & Punia, 2000) where they play a major role in the chemical and physical defense system in the seeds when exposed to environmental factors such as oxidative stress during generation of reactive oxygen species and microbial interactions thus contributing to antioxidant and antimicrobial activity (Troszynska *et al.*, 2002). From the results, also it has been documented that green gram contain high level of total phenolics than golden gram. This is in purview with available literature that darker coloured legume grains tend to contain higher concentrations of phenolic compounds than lighter coloured grains. Chang *et al.* (1994) reported higher concentrations of phenolic compounds in coloured cowpea varieties than the white varieties. The darker coloured seed coats of lima beans, pigeon peas, African yam bean and jack bean were found to contain significantly higher tannin content than the lighter coloured seed coat (Oboh *et al.*, 1998).

It has been estimated that there are approximately 8,000 naturally occurring phenolic compounds (Luthria *et al.*, 2006). Polyphenols can be classified into two major groups: (1) Phenolic Acids and (2) Flavonoids. Because phenolic acids exist in multiple forms, the polarity of each phenolic acid can vary significantly. This has lead to difficulty in developing a uniform extraction method for different phenolic acids from varying matrices. Methanol infusion and supercritical fluid extraction with CO_2 are the two most popular methods for estimation and isolation of polyphenols. However most preferred solvent used for extraction and estimation of plant phenolics is methanol. Phenols are very susceptible to enzymatic oxidation and phenolic material may be lost during isolation procedures, due to the action of specific 'phenolase' enzymes present in all plants (Harborne, 1998). Extraction of the phenols from plants with boiling alcohol normally prevents enzymatic oxidation occurring and so many laboratories practice it routinely. One of the most distinctive properties of phenols is their weakly acidic character (pKa~10). They form salts with strong bases

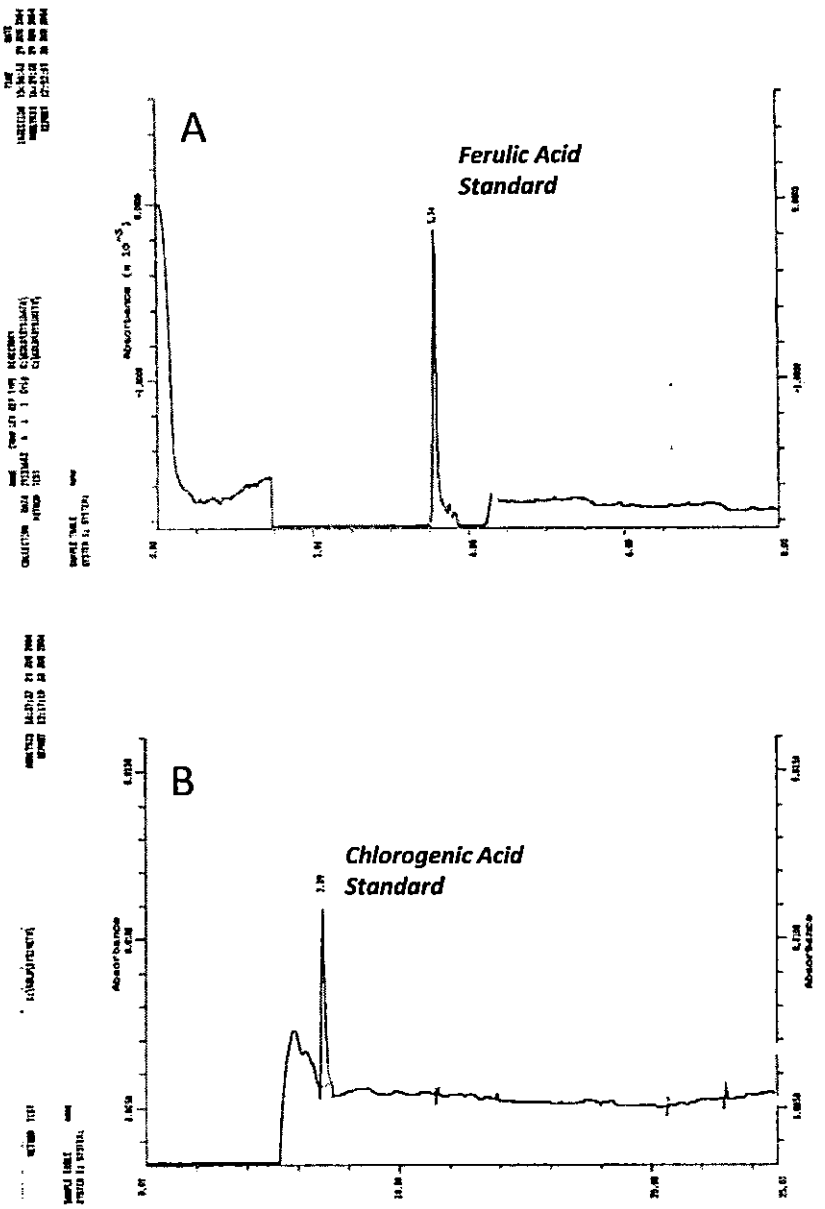

Figure 10.1 Capillary Electrophoretogram of different phenolic acid standards **(A)** Ferulic Acid **(B)** Chlorogenic Acid

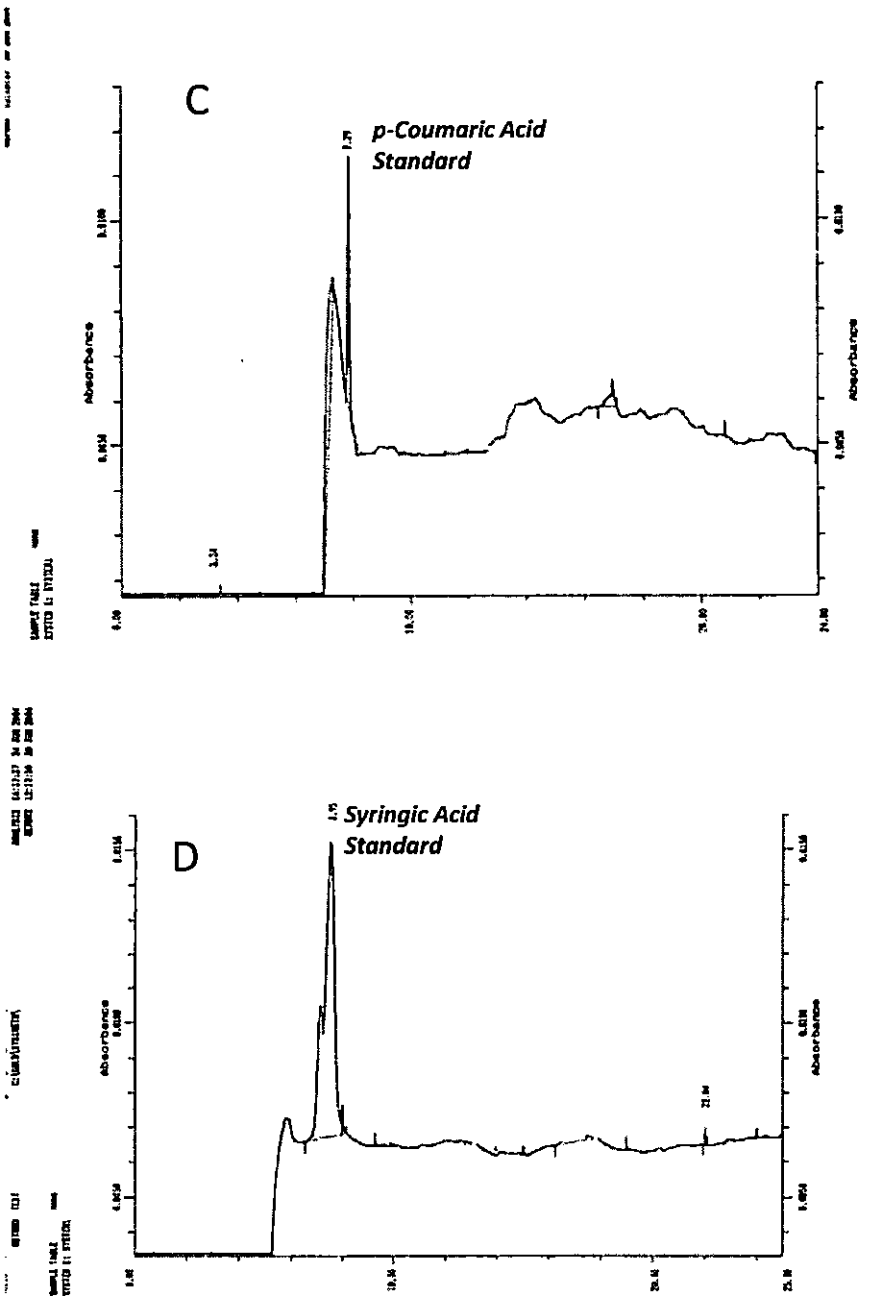

Figure 10.1 Contd. (C) ρ-Coumaric Acid **(D)** Syringic Acid

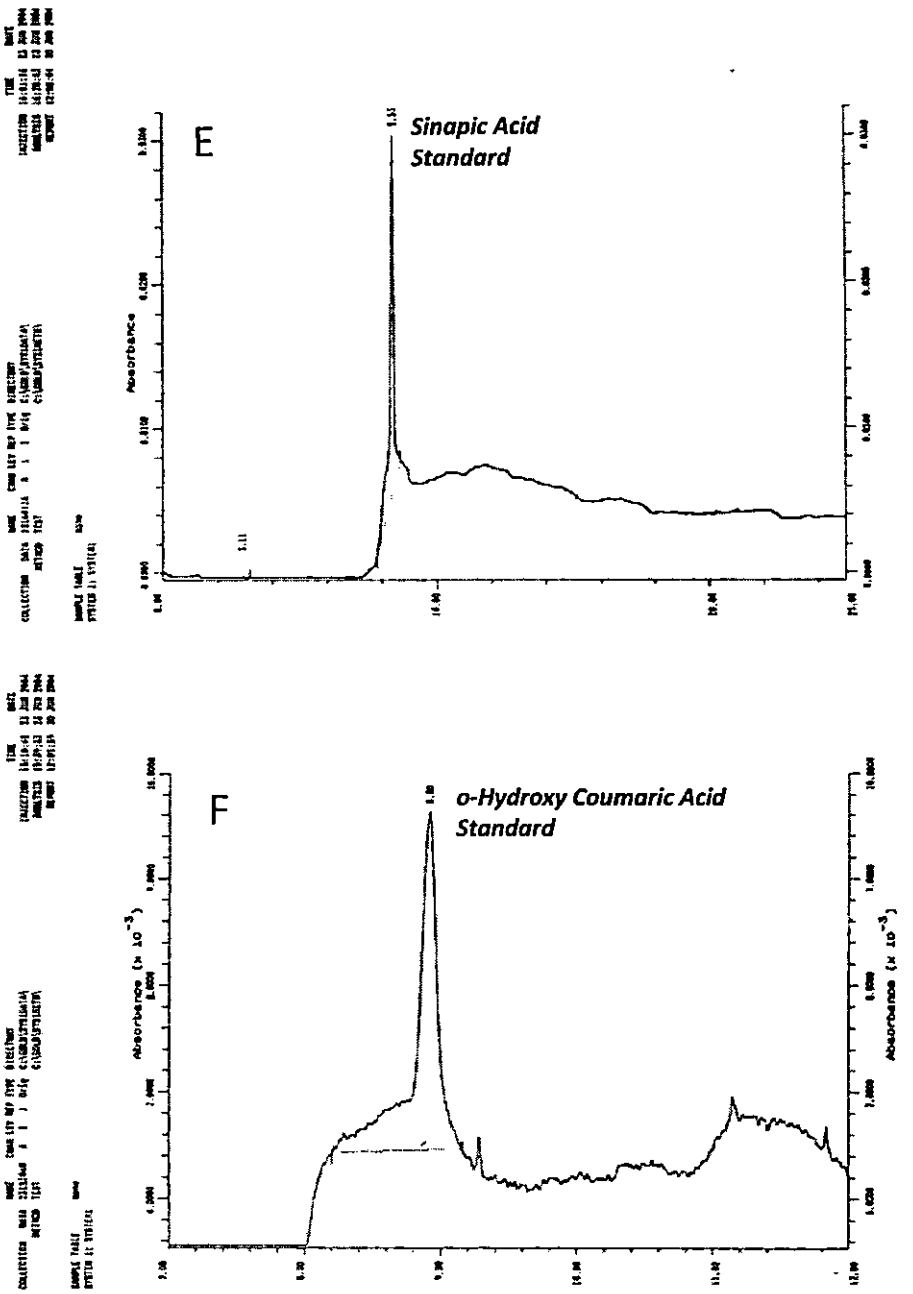

Figure 10.1 Contd. (E) Sinapic Acid **(F)** *o*-Hydroxy Coumaric Acid

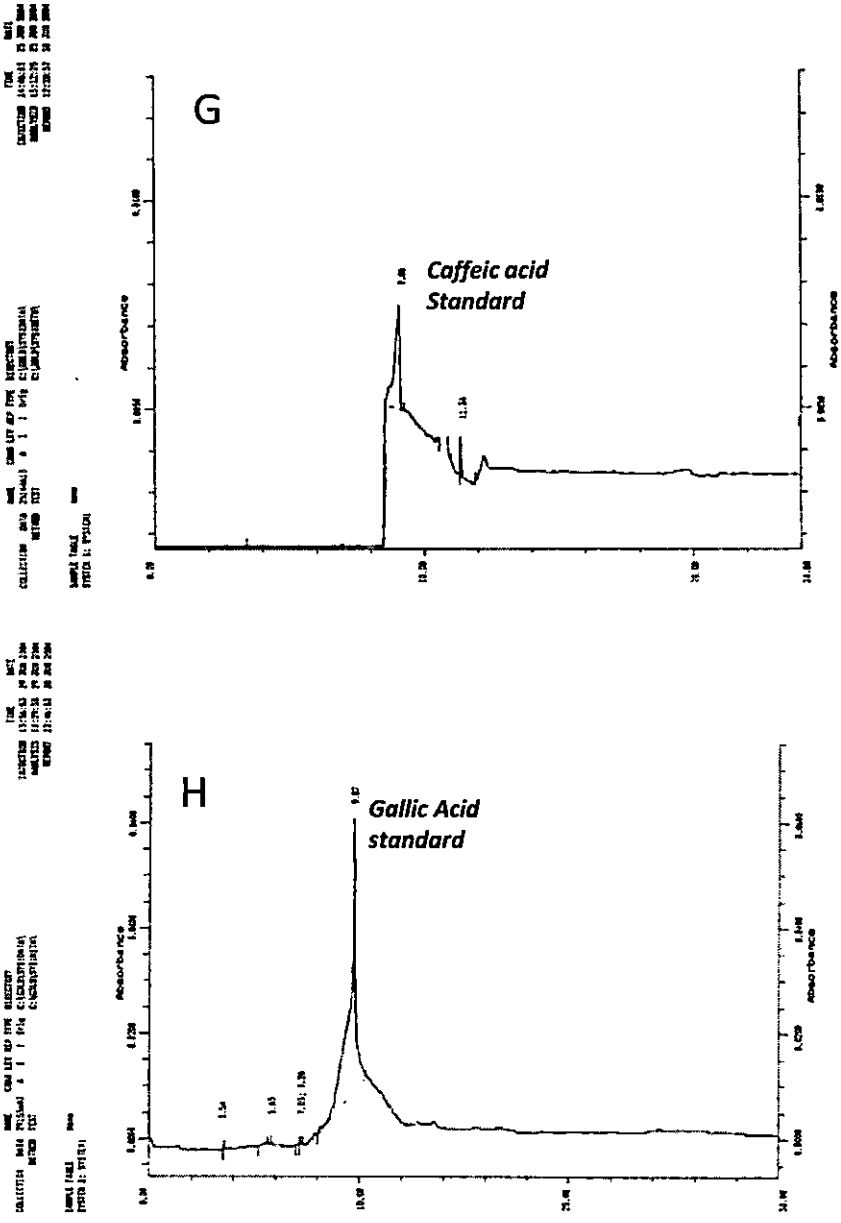

Figure 10.1 Contd. (G) Caffeic Acid **(H)** Gallic Acid

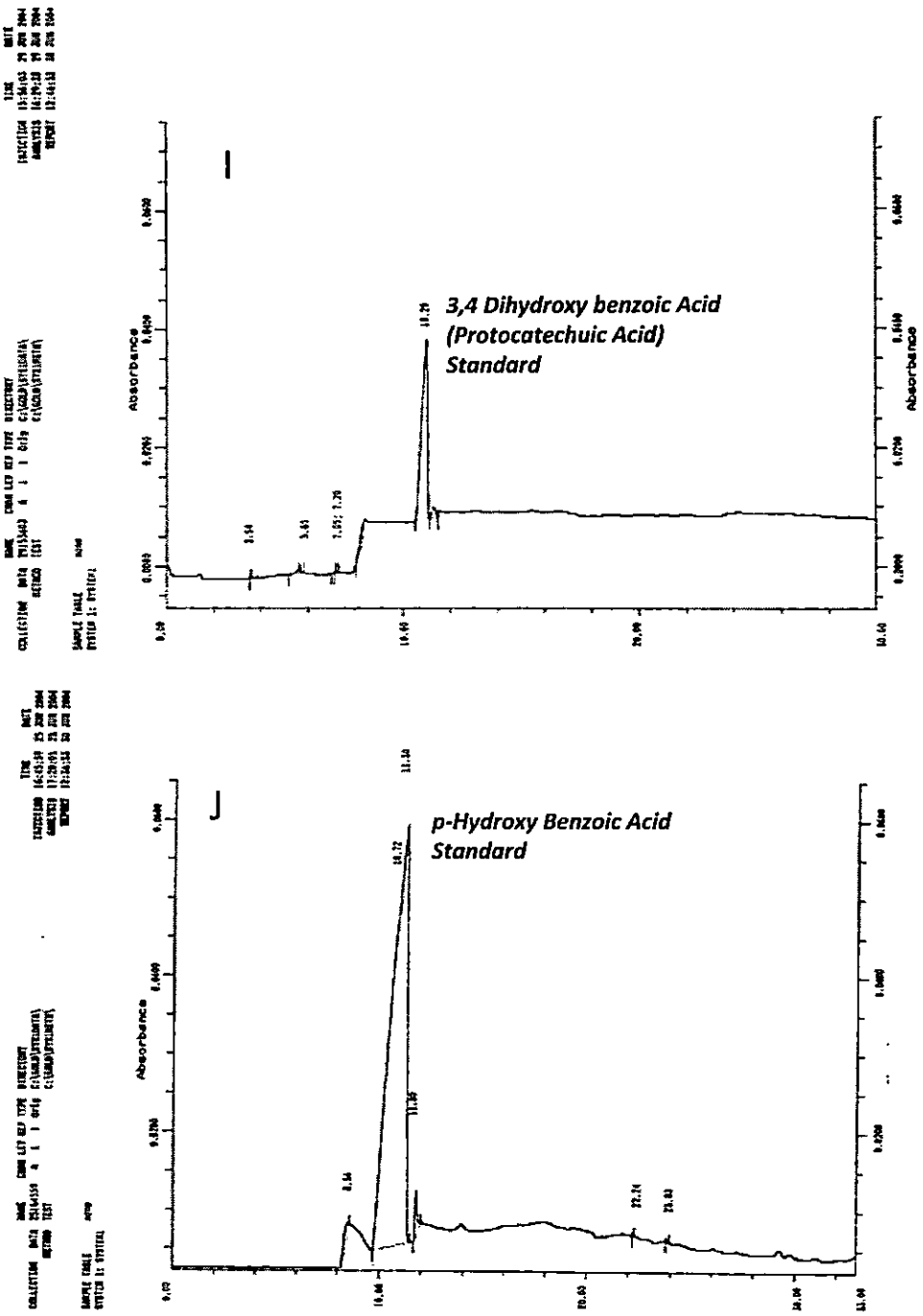

Figure 10.1 Contd. (I) 3,4-Dihydroxy Benzoic Acid
(J) *ρ*-Hydroxy Benzoic Acid

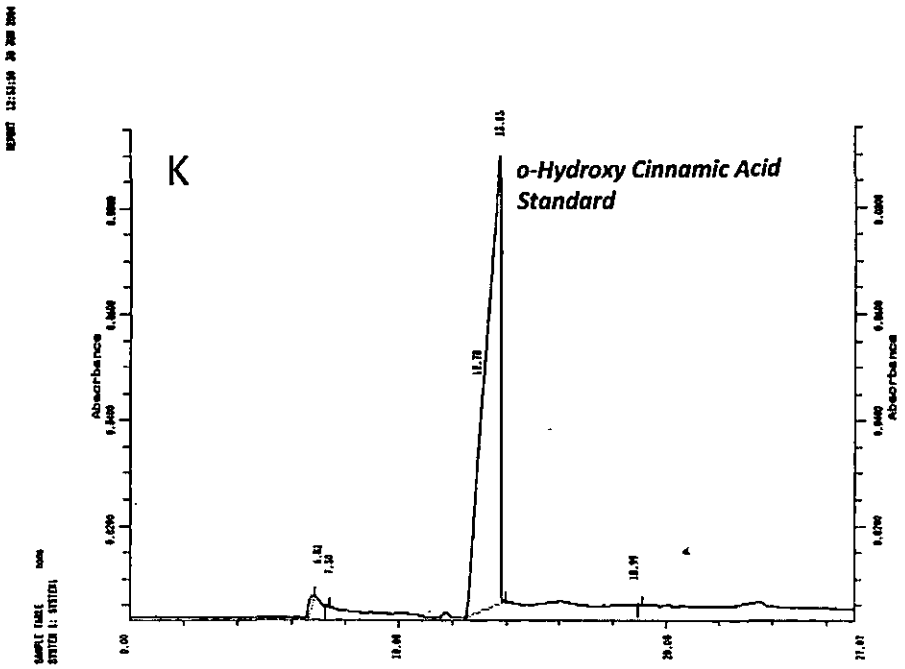

Figure 10.1 Contd. (K) *o*-Hydroxy Cinnamic Acid

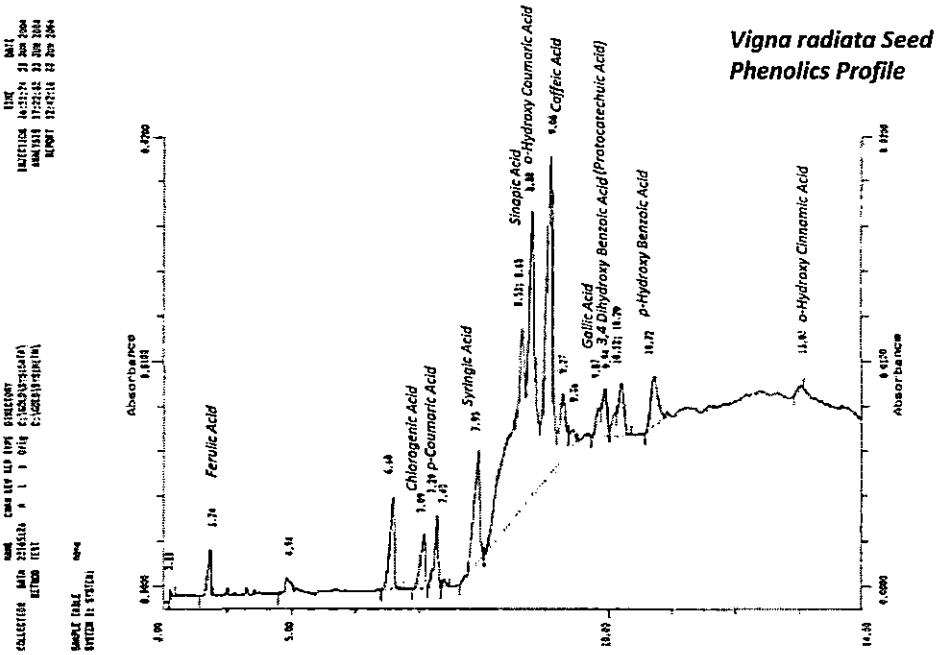

Figure 10.2 Capillary Electrophoretogram of different phenolics in seeds of *V. radiata*

but not with the corresponding carbonates. Many naturally occurring phenols contain, in addition, other functional groups attached directly to the nucleus or to the side chains. The separation of phenols from plant materials is based on their weak acidity and solubility in organic solvents immiscible with water. Thus phenolic acids are extracted from ethereal solution with water solutions of weak alkali hydroxides. The phenolates may then be decomposed by bubbling in CO_2 and the phenols re-extracted into ether. An alternative method is to extract the original ethereal solution first with 2% $NaHCO_3$ for separation of phenolic carboxylic groups and then with dilute (5%) NaOH to recover phenols. Here also same principles of alternative methods have been followed for isolation and separation plant phenolics. For quantification of different phenolic acids present in legume seeds, High Performance Liquid Chromatography and capillary Zone Electrophoresis are the reliable methods. Reverse phase HPLC using C_{18} column and solvents of water-methanol-acetic acid (12:6:1) and water-acetic acid-n-butanol (342:1:14) respectively, has successfully resolved mixtures of phenolic acids and phenolic aldehydes (Harborne, 1982). Nine different phenolic acid peaks were also identified by Mokgope (2006) through HPLC C_{18} column from cowpea seed coats. Recently Capillary Zone Electrophoresis methods have been developed by several authors for the separation of polyphenolic antioxidative compounds (Vaher and Koel, 2003). The basis of separation in CE is essentially identical to other electrophoretic techniques and its major potential is the versatility in the separations due to its various modes of operation. Figure 10.1 and Figure 10.2 shows the Capillary Electrophoretogram of different phenolic acid standards and seeds of *Vigna radiata* green variety at 214 nm detection wavelength. The profile of chromatogram of *Vigna radiata* showed the presence of various free phenolic acids (Table 10.1). The majority of phenolic acids exist in plants as structural components of the plant such as cellulose, proteins, lignin (Andreasen *et al.* 2000) with only a minor existing in the free form (Robbins, 2003). The insoluble bound phenolics can only be extracted by organic solvent after saponification (Krygier *et al.* 1982; Liyana-Pathirana *et al.*, 2006) or by enzymatic treatment (Landbo & Meyer, 2001) in order to break the ester linkages and release the phenolic acids (Robbins, 2003). So the identified phenolic acids in this work only after solvent extraction and fractionation are principally free phenolic acids. A little amount of available phenolic acids were particularly trapped in capillary electrophoresis as shown from electrophoresis profile. This is in agreement with work done by Cai *et al.* (2003), where a very small

proportion of cinnamic acid derivatives such as ferulic and *p*-coumaric acids may occur in free form and may be extracted with organic solvents such as methanol.

Table 10.1 Free phenolic acids content of crude phenolic extracts (CPE) from seeds of *Vigna radiata* Wilczek.

Phenolic Acids	*Retention Time*	*Peak Area Percentage*	*Concentration (mg/100g CPE)*
Ferulic Acid	3.74	1.44	0.22 ± 0.06
Chlorogenic Acid	7.09	2.61	0.40 ± 0.05
ρ-Coumaric Acid	7.29	2.70	0.42 ± 0.08
Syringic Acid	7.95	6.43	1.01 ± 0.41
Sinapic Acid	8.53	14.73	2.29 ± 0.27
o- Coumaric Acid	8.80	17.50	2.73 ± 0.35
Gallic Acid	9.87	1.22	0.19 ± 0.01
3,4 Dihydroxy Benzoic & Caffeic Acid	10.12	0.97	0.15 ± 0.04
ρ-Hydroxy Benzoic Acid	10.72	3.36	0.52 ± 0.08
Vanillic Acid	13.03	0.20	0.03 ± 0.01

Means ± SD within the last column with different letters are significantly different ($p<0.05$)

The phenolic components that were traced from the seeds of *Vigna radiata* contained free phenolic acids belonging to the family of Cinnamic acid derivatives (ferulic, chlorogenic, ρ-coumaric, o-coumaric, caffeic and sinapic acids) and benzoic acid derivatives (vanillic, gallic, 3,4 dihydroxy benzoic, syringic and ρ-hydroxybenzoic acids). Of all the identified free phenolic acids, ρ-coumaric acid was found to be the most abundant phenolic acid in *Vigna radiata*. Considerable amount of sinapic, syringic and ρ-hydroxy benzoic acids were also observed in this plant sample. Phenolic acids such as 3,4 dihydroxy benzoic acid (protocatechuic acid), syringic and ferulic acids have been previously reported in *Vigna unguiculata* L.(Sosulski and Dabrowski, 1984; Cai *et al.*, 2003).

The availability and accumulation of different phenolic acids in free form depends on physiological and pathological conditions of the plants in germinating seeds. There are two possible routes of these benzoic acid derivatives. They may be

formed by the β-oxidation of C_6-C_3 hydroxy cinnamic acids, or more directly by aromatization of shikimic acid or of cyclohehane derivatives. The weight of evidence is in favour of the former route being most important biosynthetically. The actual mechanism of oxidation of the C_3 side chain was examined by French *et al.* (1976) in potato tuber tissues, and it does not appear to involve the coenzyme A ester, as might be expected in the normal β-oxidation pathway. Instead, the double bond is hydrated; there is cleavage of two-carbon fragment and the aldehyde produced is then enzymatically oxidized to carboxylic acid, the route being: $RCH{=}CHCO_2H$ » $RCHOHCH_2CO_2H$ » RCHO » RCO_2H.

The most common hydroxy cinnamic acid of plant is caffeic acid which is universal in higher plants but in *Vigna radiata* seeds the observed caffeic acid concentration is reasonably less. It may be predicted that this phenolic acid will be available in bound form in mung bean or the possibilities may also be there for conversion of caffeic into ferulic acid or higher structural polymers. Chlorogenic acid is usually present is association with caffeic acid. Besides ρ-coumaric and caffeic, two methylated acids are also very common: ferulic and sinapic. Ferulic acid, in particular, can be found in many different contexts. It has been identified as an N-acyl terminal group in a protein in barley seed, linked directly to glycine and phenylalanine. Ferulic acid can be dimerised to diferulic acid, bound to the carbohydrate of cell walls in a number of grasses (Hartley and Jones, 1976). Again, ferulic acid is present free in sugar beet and cereal seeds and is reputedly a general germination inhibitor (Van Sumere *et al.*, 1975). Long term storage particularly induces higher accumulation of free hydroxy cinnamic acids (especially ferulic acid) as observed in *Phaseolus vulgaris* (Srisuma *et al.* 1989). Hydroxy cinnamic acids and their derivatives are capable of existing in cis and trans forms, and while there is evidence that the natural forms are all trans, isomerisation inevitably occurs during extraction, and mixtures of isomers are always isolated. Artefactual oxidation in the *o*-position can also occur and caffeic acid, in particular, may be partly converted to the coumarin during isolation process; that may create the available caffeic acid in lesser extent. The enzymatic oxidation of cinnamic acid in the o-position to give o-coumaric acid is the key step in coumarin biosynthesis. The introduction of a second hydroxyl group into ρ-coumaric to give caffeic is characterized by a well known group of plant enzymes-the phenolases or polyphenol oxidases. These enzymes

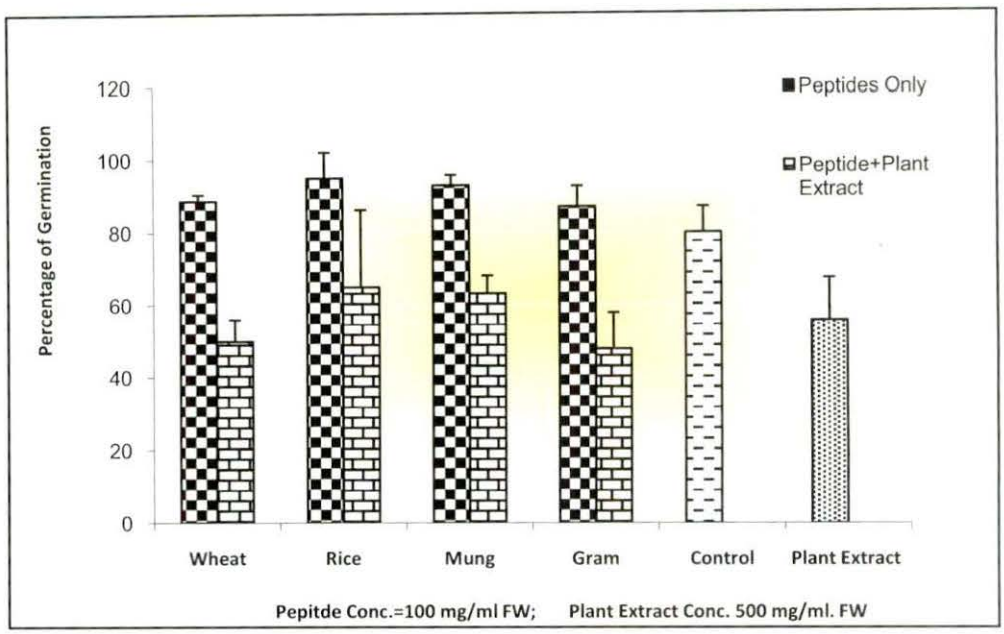

Figure 10.3 Germination percentage: peptides, plant extracts & their combination

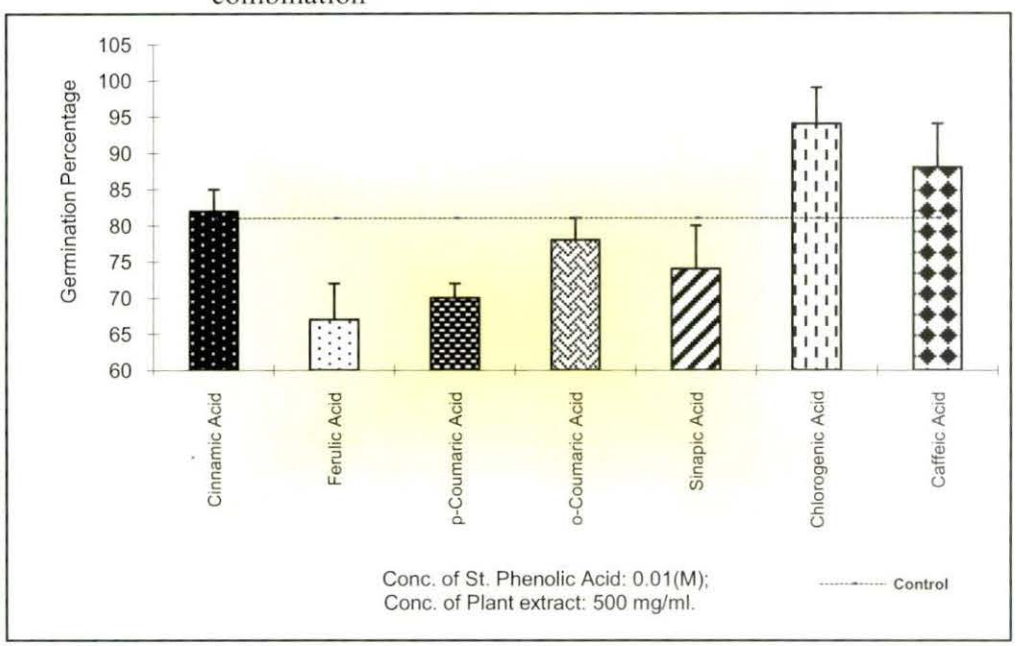

Figure 10.4 Germination precentage of mung bean in response to standard phenolic acids (cinnamic acid derivative)

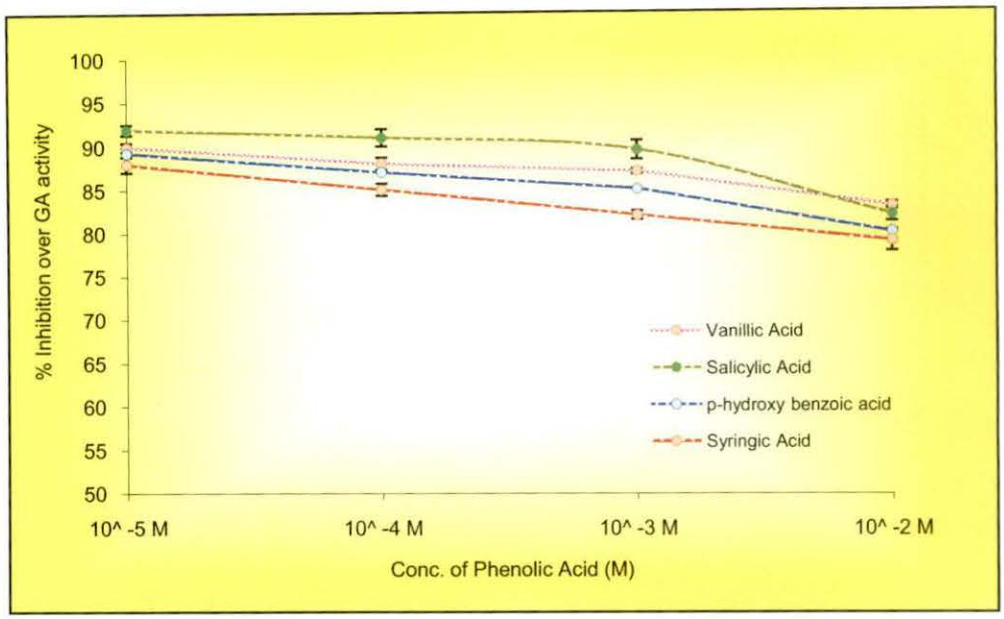

Figure 10.5 Effect of monohydroxy phenolics on germination [Benzoic Acid Derivative]

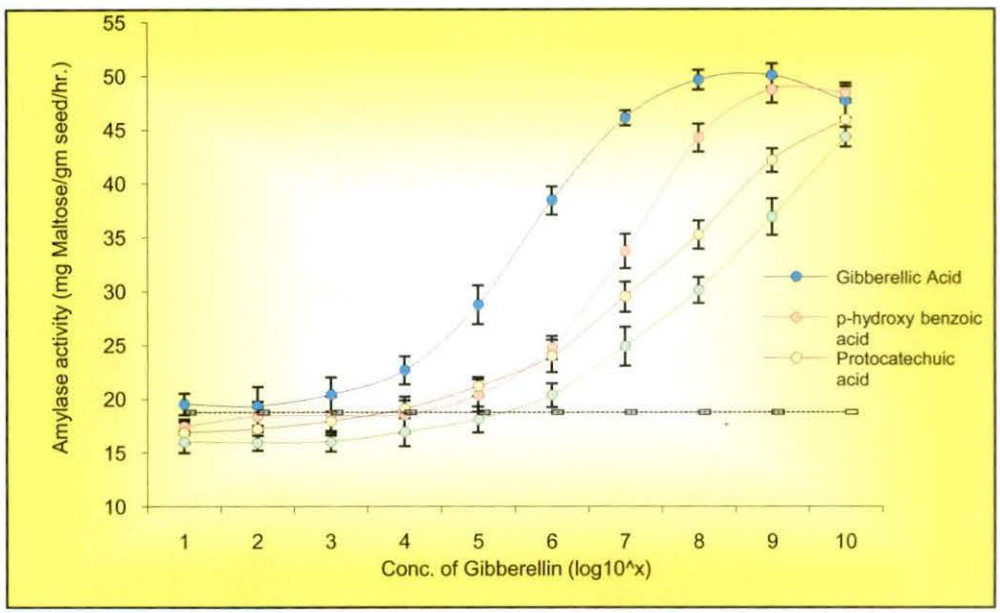

Figure 10.6 Effect of Gibberellic Acid on Amylase Induction in presence or absence of Phenolics (1 mM)

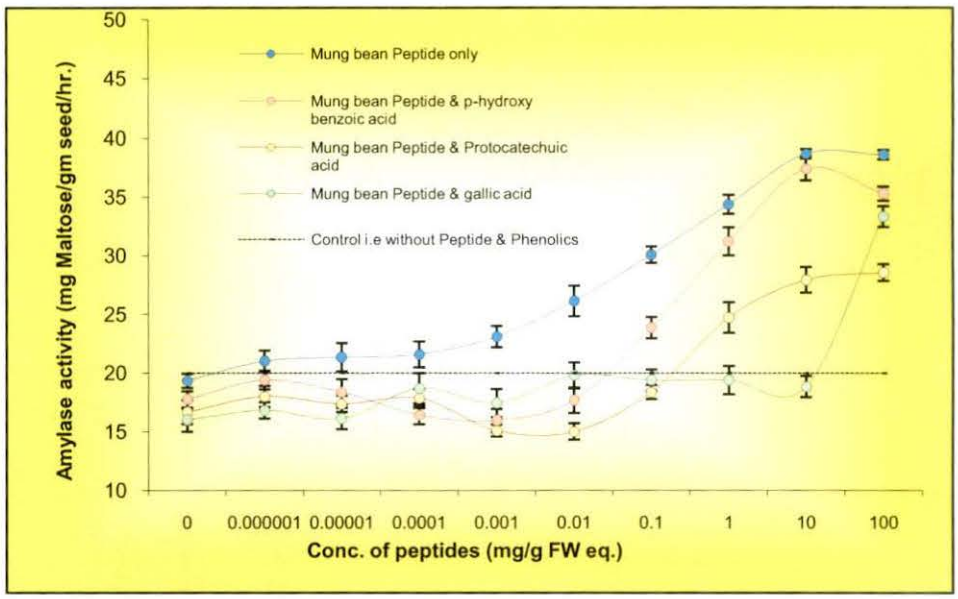

Figure 10.7 Effect of mung bean peptide on amylase induction in presence or absence of phenolics (1 mM)

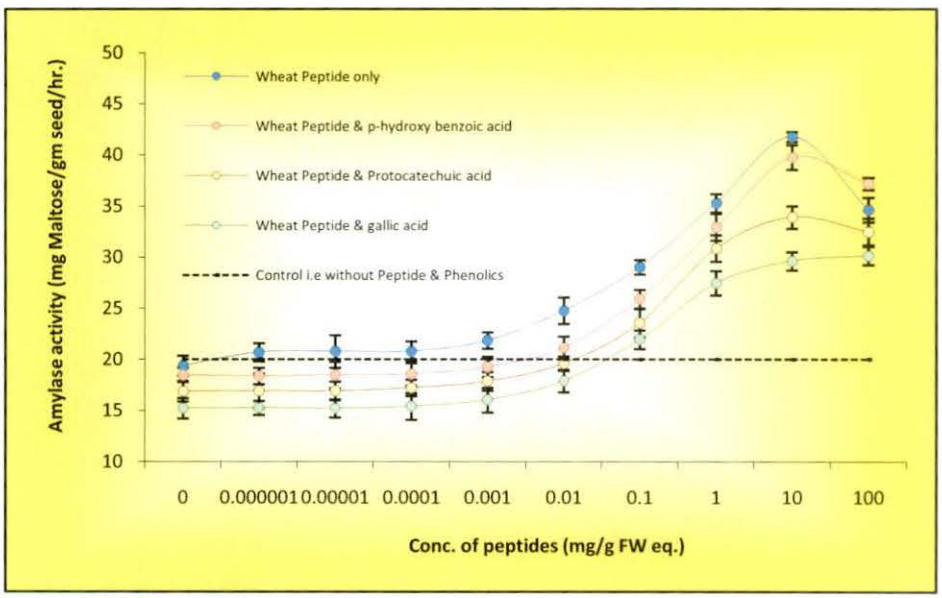

Figure 10.8 Effect of wheat peptide on Amylase Induction in presence or absence of phenolics (1 mM)

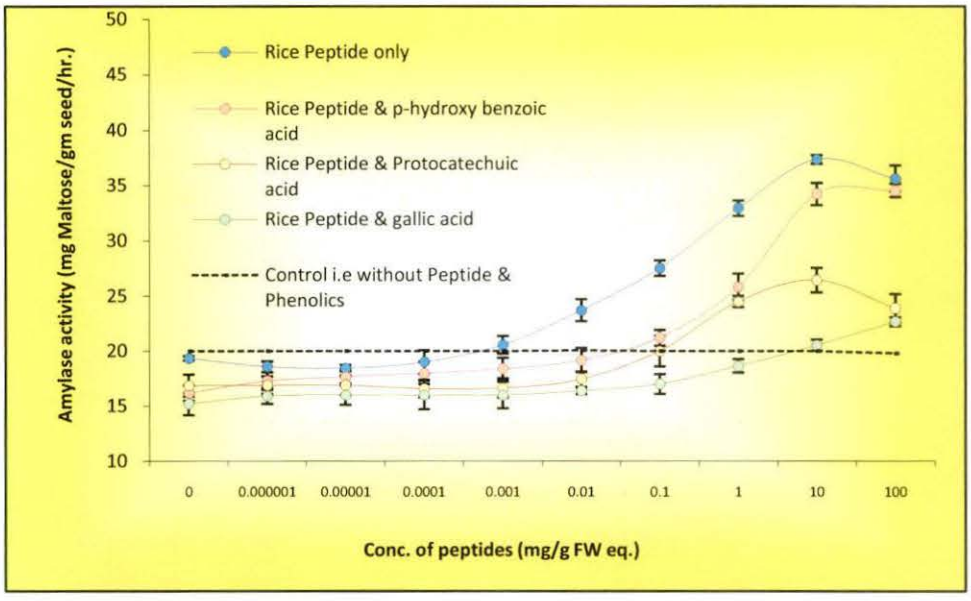

Figure 10.9 Effect of rice peptide on Amylase Induction in presence or absence of Phenolics (1 mM)

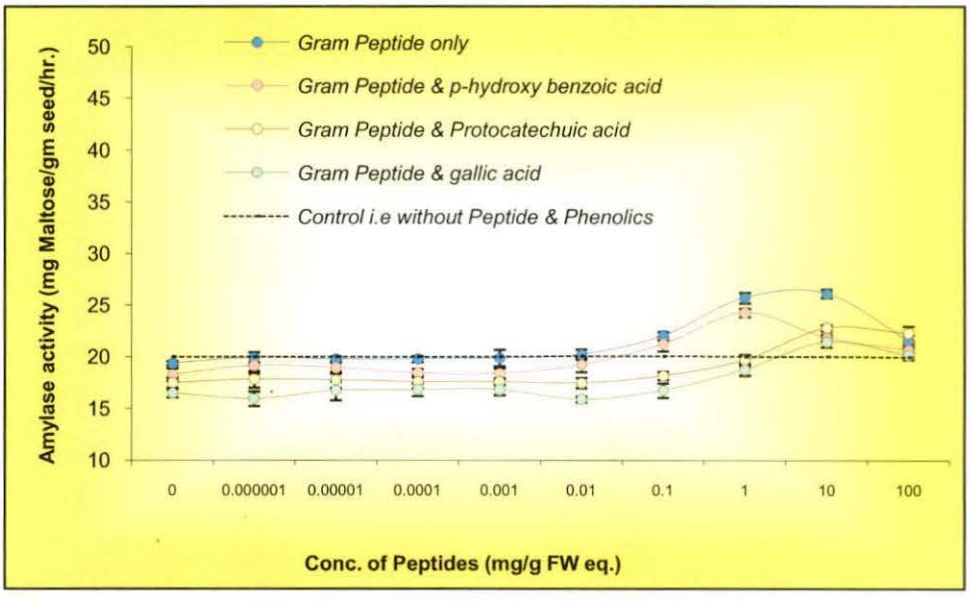

Figure 10.10 Effect of gram peptide on Amylase Induction in presence or absence of Phenolics (1 mM)

normally oxidize the caffeic acid further to the corresponding o-quinone and this then polymerizes to dark products. The reaction can be limited to the first stage in presence of ascorbic acid as reductant. One process which probably reduces the chance of phenolic compounds interfering with enzymatic reactions in the cell is polymerization. Undoubtedly, as the molecular weight of a phenolic compound increases, its transport within the cell is considerably diminished. Hydrolysable tannins are probably the most popular bioactive polyphenols which may interfere with the growth and development of seedlings. In hydrolysable tannins, glucose may be linked to phenolic groups to render the molecules more water soluble and ensure their sequestration in the vacuole. It has also been established that several tannins (hydrolysable tannin in particular) are potent inhibitors of gibberellin induced growth, whereas growth induced by indoleacetic acid was not repressed by the same. This difference in action also suggests specificity between the tannin and Gibberellic acid (Corcoran *et al*, 1972).

10.3.2 Bioactivity of polyphenols and their interaction with peptides

The results presented here shows that the germination and growth of wheat seedlings are potentially inhibited by isolated phenolic acids (Figure 10.3) and standard phenols (Figure 10.4 and 10.5). Phenols can also inhibit the biological action of Gibberellic acid and peptide induced amylase enzyme as reflected from our experiment (Figure 10.6 - 10.10). There is still considerable uncertainty in some points as to whether phenolic compounds have a physiological role in plant growth, development and metabolism during germination and post-germination phases. Even when most of the plant phenolics are not hormones themselves, they may affect the germination and growth by interaction with one or other major class of distinguished plant hormones like auxin and gibberellin (Harborne 1982). Graphical presentation clearly shows that germination processes were enhanced in response to standard Gibberellic acid like GA_3. Gibberellic acids play a role in the accumulation of seed storage proteins during maturation presumably by specifically impeding the accumulation of 12s Globulin precursors. To characterize its bioactivity we treated embryoless half wheat seeds with a solution of Gibberellic acid instead of plain water. The higher level of hormonal signal causes more synthesis of amylase. Gibberellic acids induce sudden and rapid cell growth; also stimulate the hydrolysis of starch in germinating seeds by induction of amylases. This results in a large acceleration of maltose generation. It is

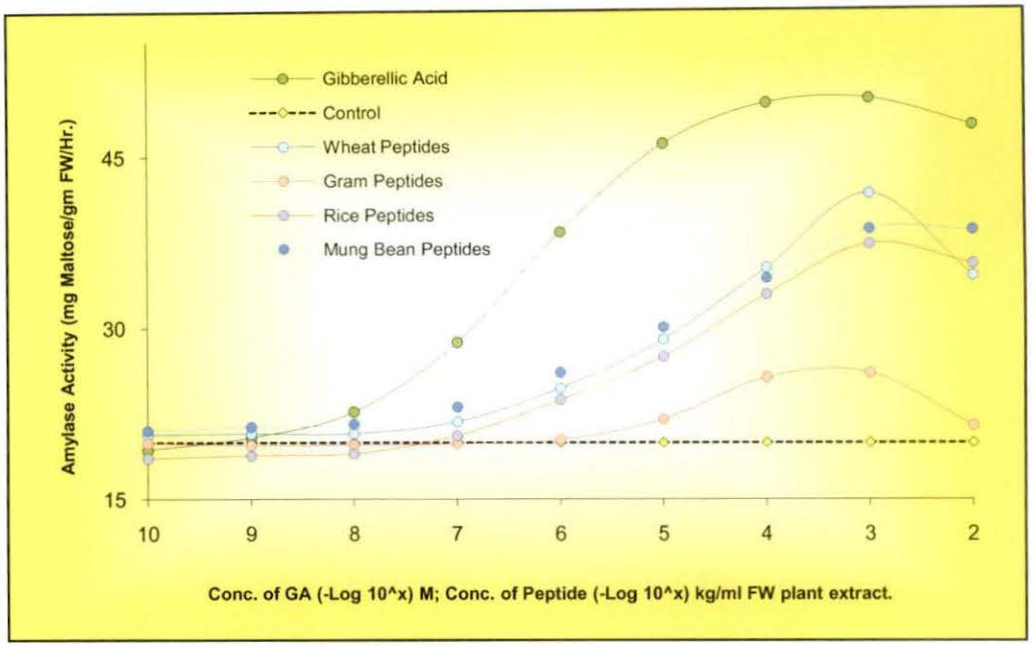

Figure 10.11 Amylase induction assay by GA3 & plant peptides

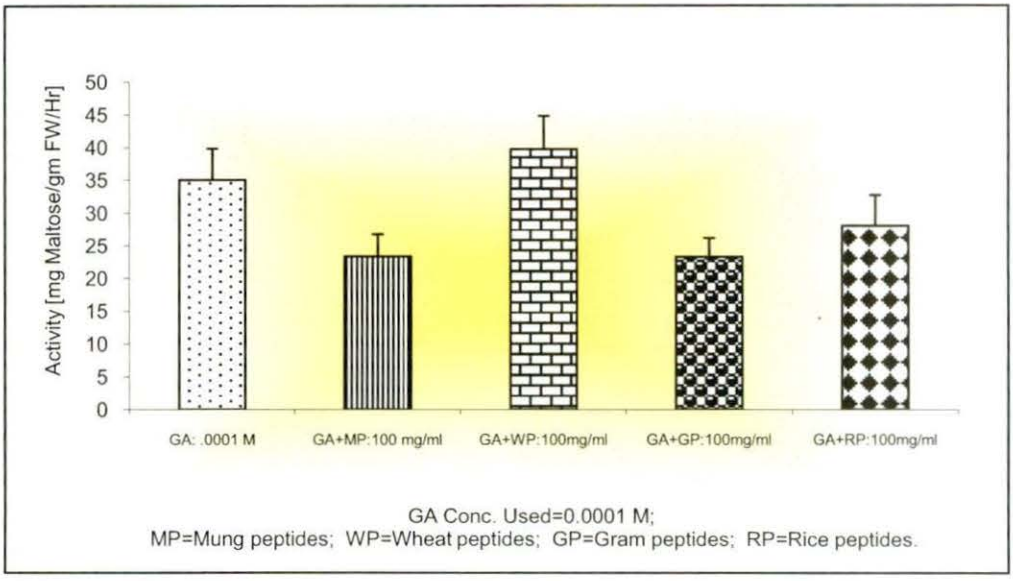

Figure 10.12 Amylase induction: gibberellic acid & peptide combination

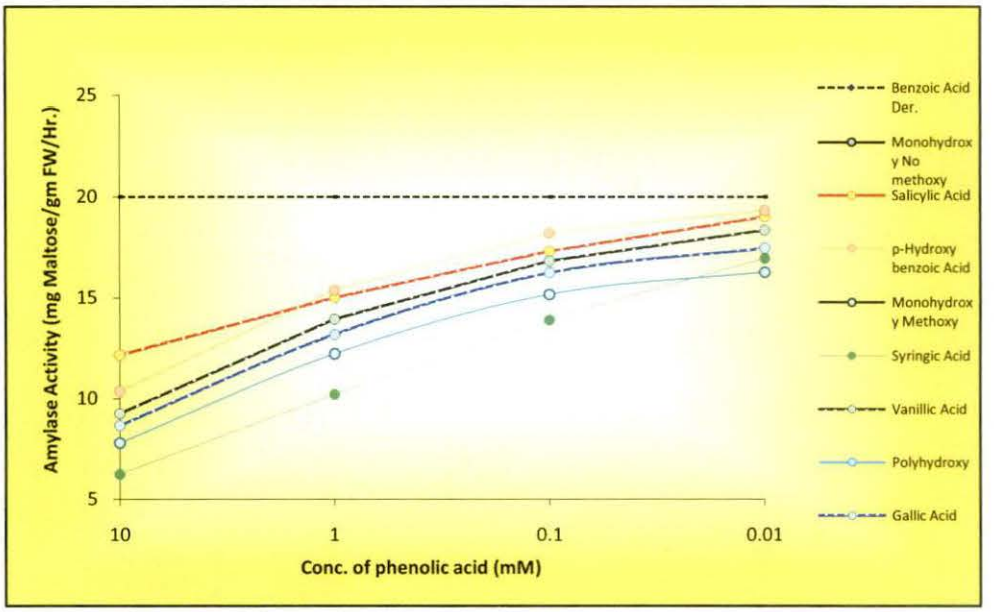

Figure 10.13 Amylase induction assay in response to phenolic acids (benzoic acid derivatives)

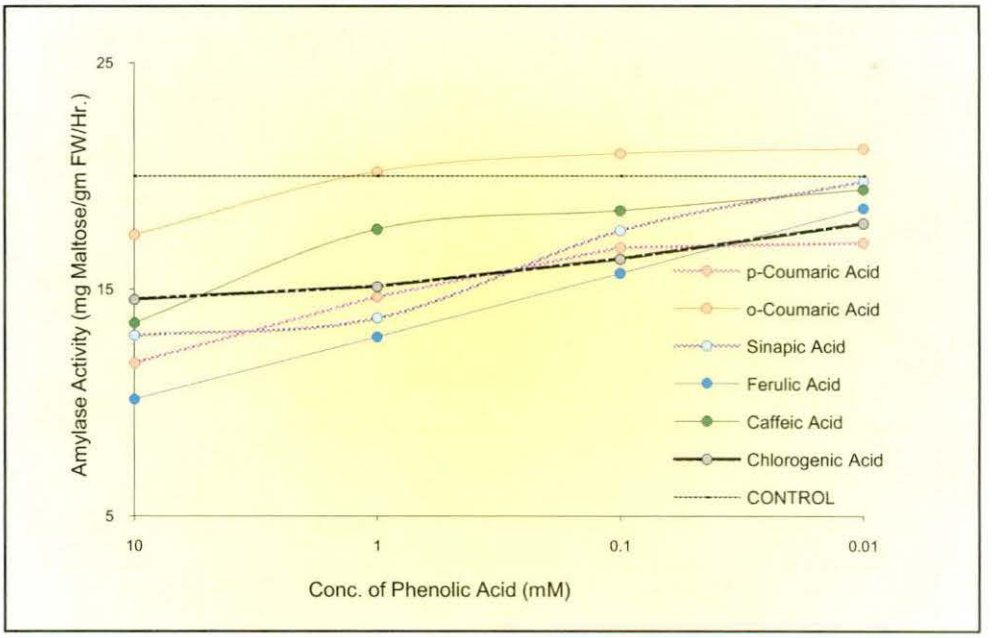

Figure 10.14 Amylase induction assay in response to phenolic acids (cinnamic acid derivatives)

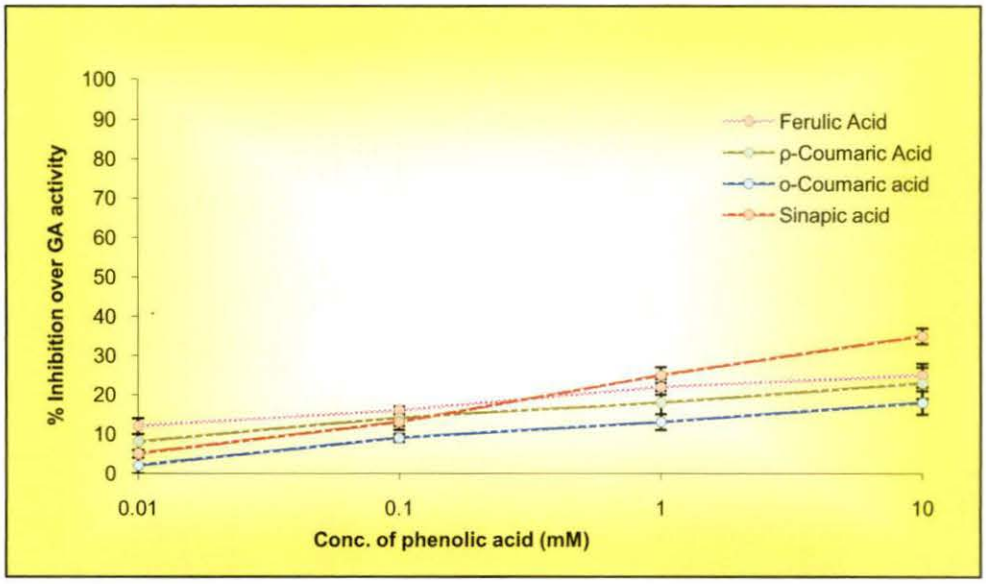

Figure 10.15 Effect of monohydroxy phenolics on amylase induced by GA3(10 μM) [cinnamic acid derivative]

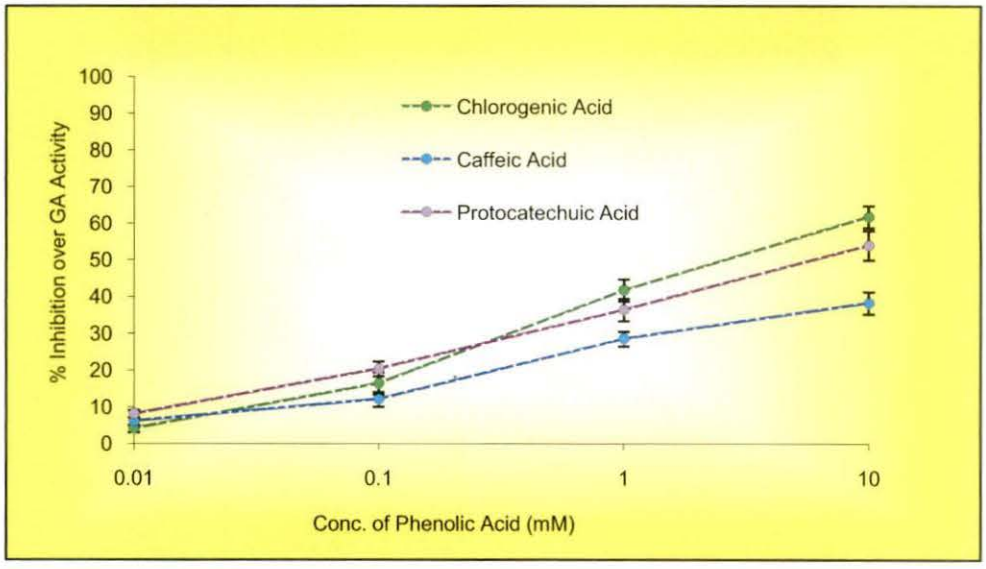

Figure 10.16 Effect of dihydroxy phenolics on Amylase induced by GA3 (10 μM)

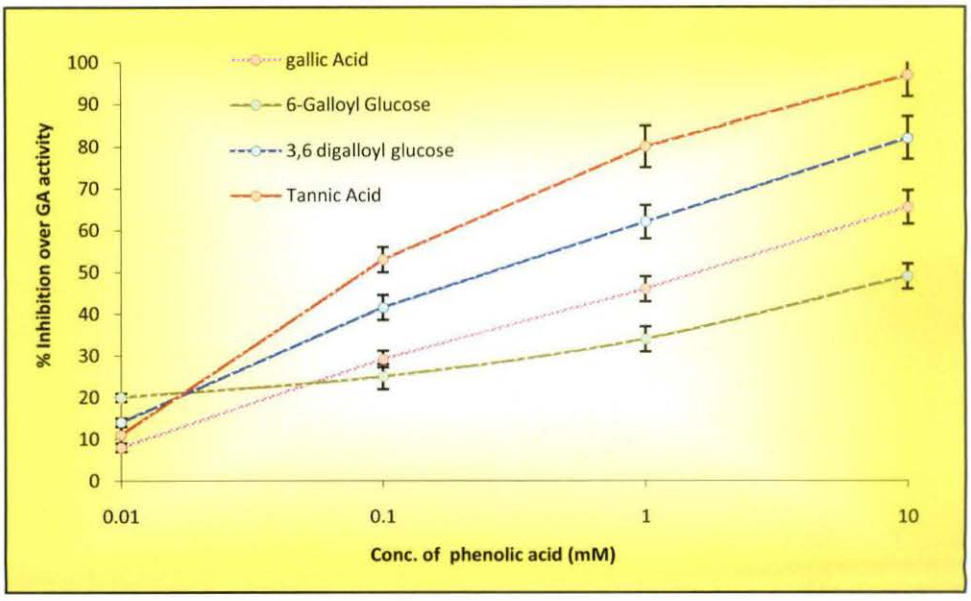

Figure 10.17 Effect of polyhydroxy phenolics on amylase induced by GA3(10 μM) [Gallic acid derivative]

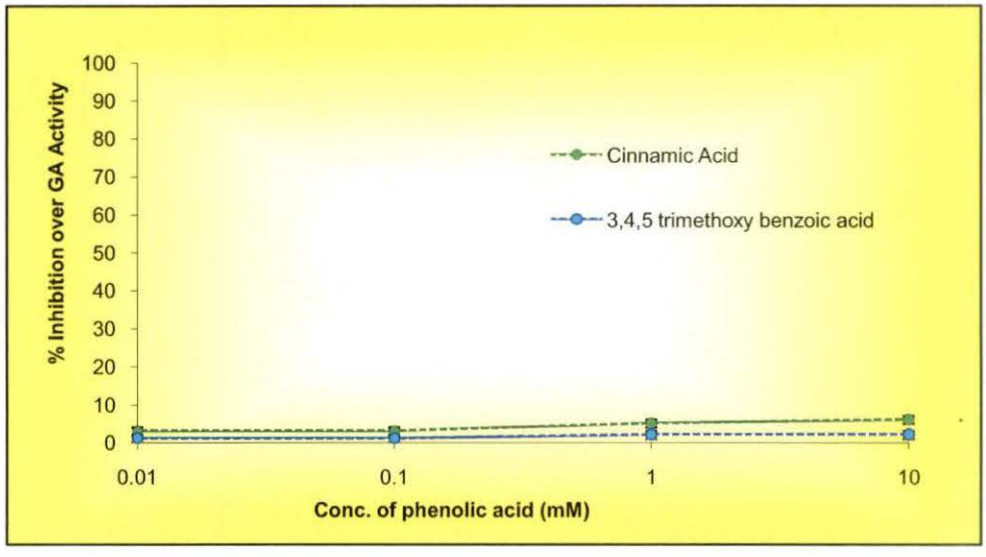

Figure 10.18 Effect of cinnamic acid & methoxy derivatized phenolates on amylase induced by GA3(10 μM)

suggested that Gibberellic acid might be involved in controlling the accumulation of protein associated with radicle protrusion and post germination processes.

In our experiments, eleven different types of standard phenolic acids and phenolic compounds extracted from seeds of *Vigna radiata* (Wilczek.) were tested for the abilities to inhibit gibberellin and peptide induced amylase syntheses in the embryoless half wheat seeds. As already stated in earlier chapters that low molecular weight peptides below 3 KDa can able to induce amylase unbiased (no species specific) regardless of its origin (Figure 10.11). These peptides could also enhance the induction of amylases in combination with gibberellic acids but synergistic responses were not observed (Figure 10.12). Phenolic acids with free hydroxyl group can successfully inhibit this specific biological action of peptides in embryoless half wheat seeds (Figure 10.7 – 10.10).

In the absence of Gibberellic acid, except *o*-coumaric acid, all different kinds of phenolic acids potentially executed minimal endogenous expression of functional amylases (Figure 10.13 and 10.14). This inhibitory action of phenolics was also observed even when half seeds treated with 10μm GA_3 (Figure 10.15 and 10.16). But most interestingly gallic acid and their various galloyl esters and glucose derivatives (including tannic acid) are most potent in this respect (Figure 10.17). The inhibitory action of phenolics were observed to be correlated with the number of free hydroxyl group associated with benzene ring, that is why the digalloyl glucose compounds were less inhibitory than those of tannic acid and gallic acid. No inhibitory actions were observed with cinnamic acid where no free hydroxyl group is present and 3,4,5-trimethoxy benzoic acid where all hydroxyl groups were substituted by methyl group (Figure 10.18). As gallic acid is one of the components of tannins (gallotannin in particular), it is presumable that tannin can also antagonize the biological action of Gibberellic acid (Corcoran *et al.* 1972). From Figure 10.15 & 10.19, it is also clear that cinnamic acid derivatives (Figure 10.15) showed lesser antagonistic action against gibberellic acid in comparison to benzoic acid derivatives (Figure 10.19).

Same picture was also observed in case of peptides isolated from wheat and *Vigna radiata*. The reversibility of the inhibitory effects was tested by adding increased amount of gibberellic acid or isolated peptides to constant amount of phenolic acids or isolated seed phenolics (Figure 10.7). This experiment was done because overcoming the response of an inhibitor by increasing the amount of promoters is consistent with the interpretation that the inhibitor and promoter are

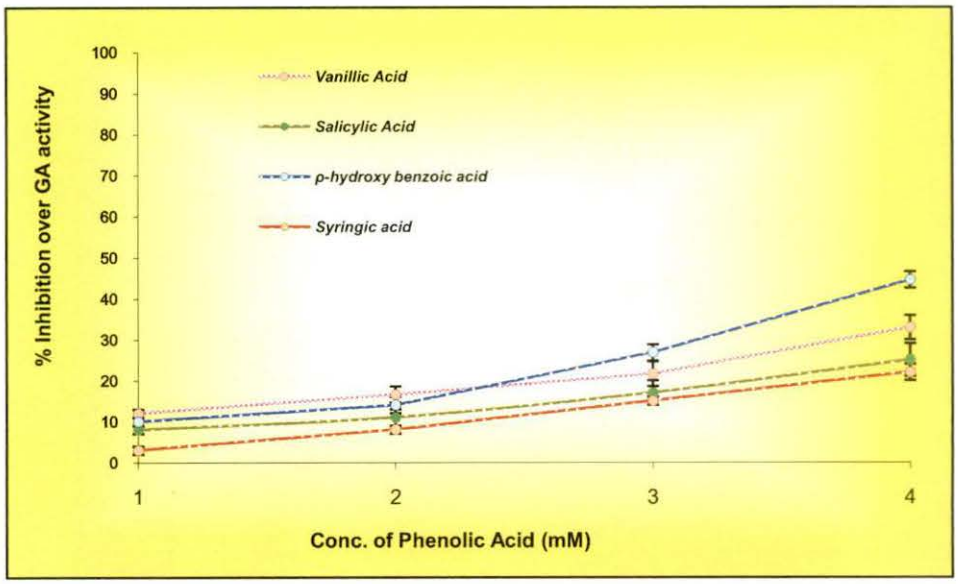

Figure 10.19 Effect of monohydroxy phenolics on amylase induced by GA3(10 μM) [*benzoic acid derivative*]

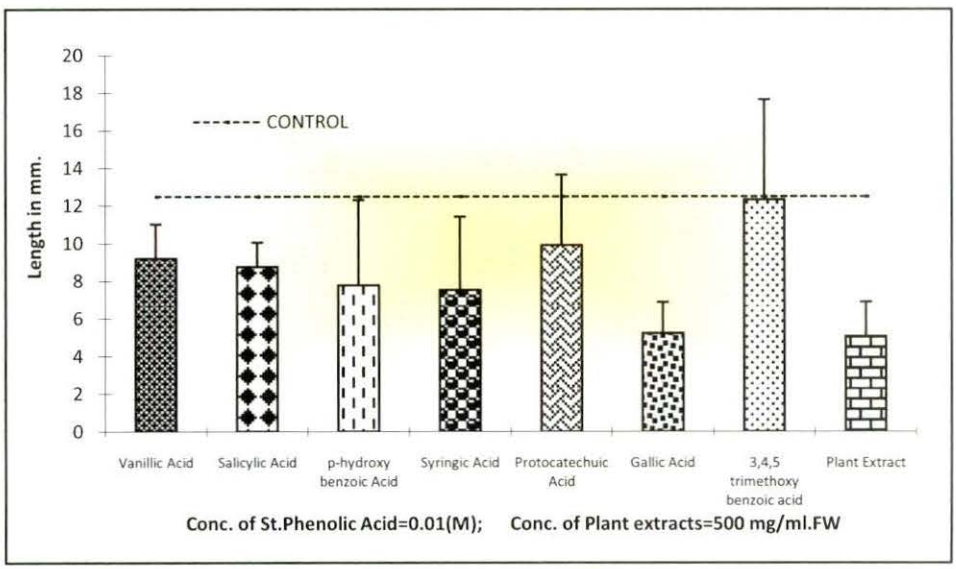

Figure 10.20 Radicle elongation: standard phenolic acids & plant extracts (*benzoic acid derivative*)

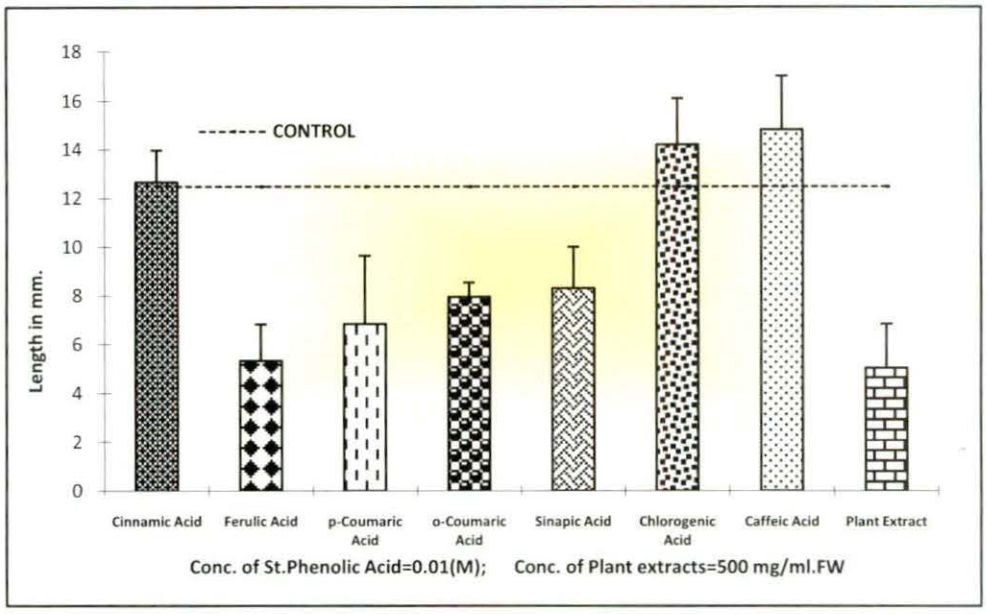

Figure 10.21 Radicle elongation: standard phenolic acids & plant extracts (*cinnamic acid derivative*)

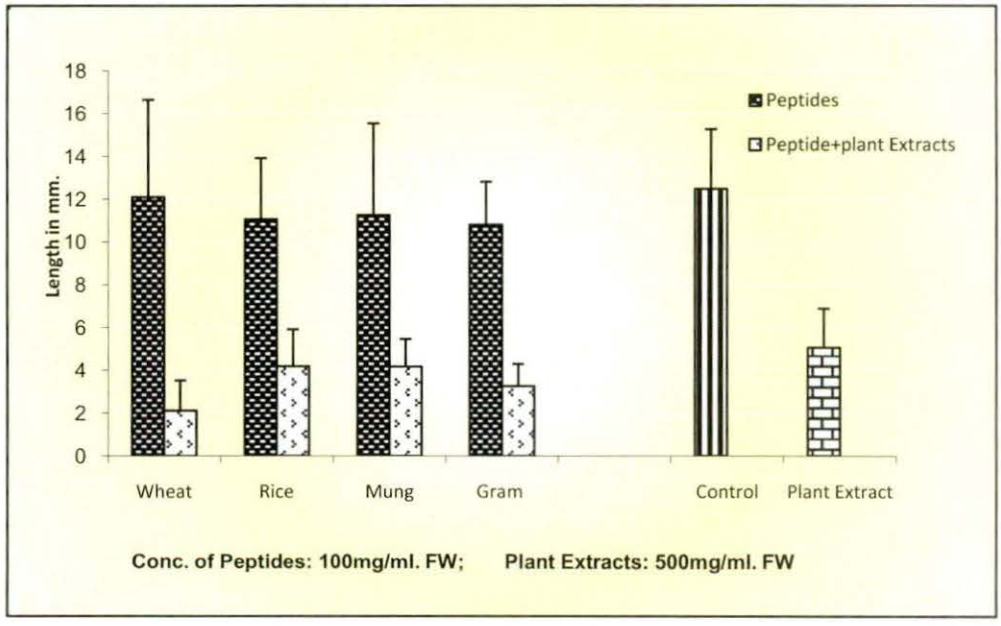

Figure 10.22 Radicle emergence: peptides, plant extracts & their combinations

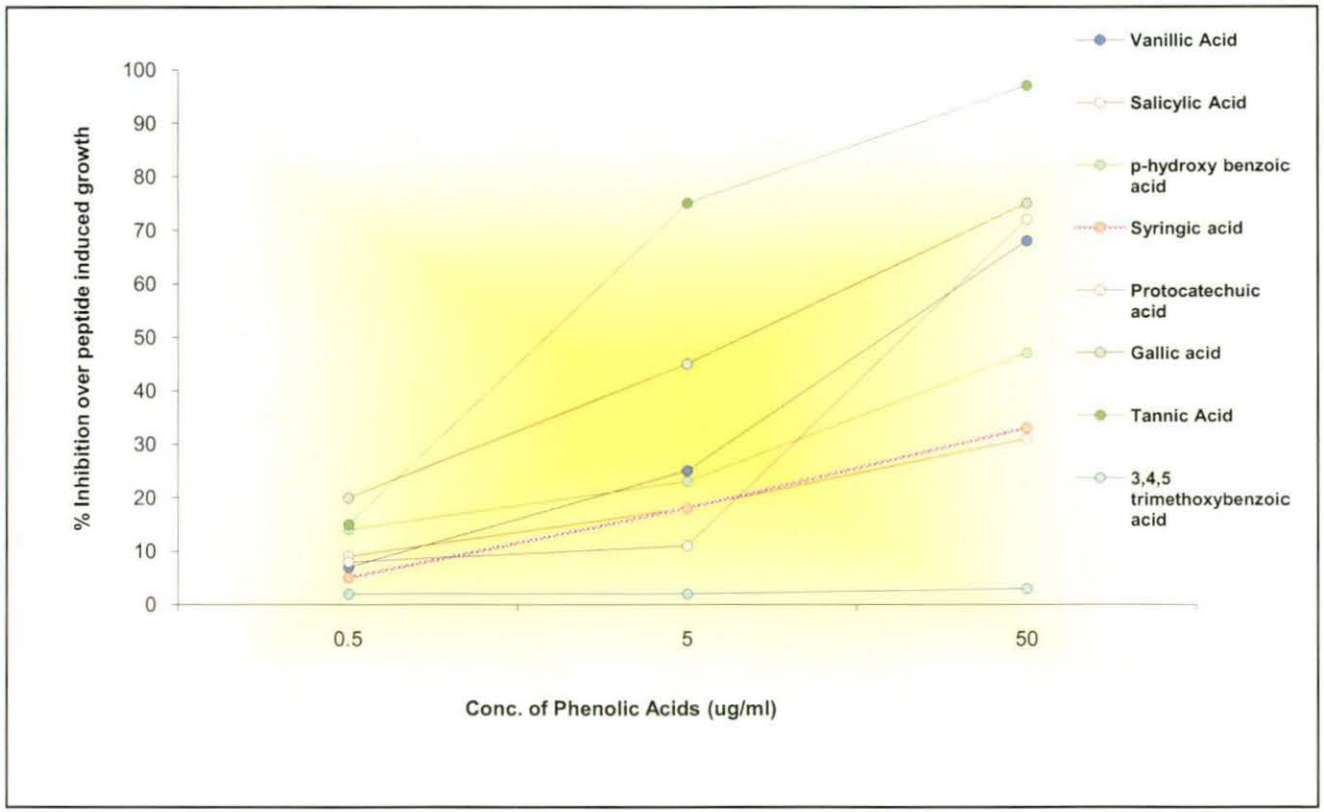

Figure 10.23 Effect of phenolic acids (benzoic acid derivative) on shoot growth induced by Mung bean peptides

involved in the same system. A constant amount of 1 mM of phenolics was used throughout the experiment. The amount of Gibberellin and peptide used ranged from 10^{-1} to 10^{-10} M and 10^{-6} to 10^{2} mg/g of fresh weight equivalent respectively. The inhibitory activity of three different types of phenolic acids (viz. mono-, di- and trihydroxy phenolates) was reversed by both Gibberellic acid as well as different isolated peptides. But here also the highest concentrations of inducers were required for recovering the inhibition of gallic acid which was followed by protocatechuic acid and ρ-hydroxy benzoic acid. The data presented here confirmed that the phenolic acids (Figure 10.20 and 10.21), peptides, gibberellic acid and their different combinations not only affect germination but also alter seedling morphology (radicle and shoot length) (Figure 10.22). Peptide can able to enhance both radicle and shoot length elongation of seedlings of chickpea. This is in agreement with recent works where low molecular weight peptides like CLAVATA-3 can able to modulate physiological activity like meristems determination, apical-basal polarity and radial patterning observed in *Arabidopsis* and other angiosperms. Even when most phenolics are not hormones themselves, they may affect plant growth by interaction with one or other major classes of plant hormones like auxin. It is well established fact that mono-hydroxy cinnamic acid uniformly augment the activity of IAA oxidase and thus potential inhibitory effect on growth whereas cinnamate with more than one hydroxyl group can able to enhance growth by inhibiting the oxidase activity. In our experiment it is observed that almost all kind of phenolic acids inhibited wheat and mung bean peptide induced growth of the shoots and most prominent inhibitory action was observed in case of phenolics derived from benzoic acid (Figure 10.23). The phenolic components isolated from seed of *Vigna radiata* also prominently exhibited peptide induced growth inhibition (Figure 10.22). The predominant component of isolated phenolics includes sinapic acid, syringic acid and ρ-coumaric acid where only one free hydroxyl group is associated with benzene ring. So the inhibitory action played by isolated phenolics may be due to the influence of monohydroxy phenolics actually present in the seed coat of *Vigna radiata*. Identical pattern of growth of radicle was observed under different treatment of standard phenolic acids and plant extracts (Figure 10.20 & 10.21). Gallic acid and ferulic acid were shown to be maximum active for inhibition of radicle elongation, also the isolated phenolics exhibited potential inhibitory activity. But altered response was observed in case of chlorogenic and caffeic acid (with more than one free hydroxyl group) where partial

enhancement of growth of radicle was documented from our experiment. Plant peptide itself has no significant role on radicle emergence and elongations as noticed from our experiment, but when the plant peptides were combined with extracted seed phenolics, significant retardation of growth of radicle was observed in all cases. (Figure 10.22)

One interesting observation comes out from our experiment that embryoless half wheat seeds when treated with peptides can significantly induce amylase. Percentage of germination of mung beans was not significantly improved as such after the same applications (Figure 10.3). Extracted plant phenolics as well as standard phenolic acids generally inhibited the process of germination, when applied independently or in combination with gibberellic acid or peptide inducers. Few exceptions were there as for example protocatechuic acid, chlorogenic acid and caffeic acid where germination is enhanced when applied alone (Figure 10.4). It is already stated that amylase induction in embryoless half wheat seed is drastically inhibited after application of different phenolic acids in free form or in combination with inducers (Figure 10.8). So the apparent paradox of the behaviour of some phenolic acid in embryo containing and embryoless half seeds can be resolved from the observation of combined application of phenolic acids either with peptide or Gibberellic acid where inhibition was drastic and significant in all cases. From these observations it is probable that phenolic acids cannot directly inhibit the signal of germination but practically quench with the activity of Gibberellic acid or peptides.

From the results discussed above it can be interpreted that germination is a multicomponent based interaction processes where degradation of stored starch is essential for proper growth and development of the embryo. During germination processes, the hydrolysis and mobilization of these nutrients are dependent on temporal accumulation and dynamic shifting of inducers and inhibitors respectively. Seeds integrate many intrinsic signals to control germination (Koornneef *et al* 2002). The mechanism by which these signals are integrated is still unknown in several cases. As most of the works are concentrated on gibberellic acid and its antagonizing hormone ABA, less attention has been paid to phenolic components and peptides which may couple with several intermediates of different signal transducing processes. Phenolic compounds are some of the most widespread metabolites and play a significant role in germination, growth and development (Makoi and Ndakidemi, 2007). Growth-inhibitory compounds of agricultural importance have recently

received considerable research attention (Siqueira *et al* 1991, Inderjit 1996). These compounds include phenolic compounds with allelopathic characteristics (Schenk *et al* 1999). Studies have shown that these chemicals include simple phenols, phenolic acids derived from benzoic and cinnamic acids, coumarins, flavonoids, isoflavonoids, tannins and a variety of phenolic conjugates. Their presence and accumulation in the soil may reach the threshold concentrations for inhibition of pre-emergence seed germination or post germination, growth and nutrient absorption. Different phenolics concentrations have been reported to inhibit seed germination and seedling growth in legumes (Beier *et al* 1983, Zaynoun *et al* 1984, Devi and Prasad 1992). It has been observed that phenolic compounds significantly reduce the chlorophyll content, soluble protein and nitrate reductase activity. Enhanced contents of thiobarbituric acid reacting substances (TBARS) and hydrogen peroxide were documented with phenolic treatments. Phenolics can able to enhance the activity of different antioxidant enzymes like Superoxide Dismutase, Catalase and Peroxidase below its threshold level. But at higher concentrations phenolic compounds significantly reduced the antioxidant activity over control. So the susceptibility at higher concentrations may be attributed to reduced antioxidant enzyme activity and more membrane damage (Djanaguiraman *et al.*, 2005). Polyphenols of mung bean has low protein precipitating capacity, relatively high flavonol levels and were concentrated in the seed coat. Leakage of polyphenols from mung bean seeds has started immediately after imbibition. Mung bean sprouts had 36% less polyphenols after 48 hours of germination (Barroga *et al.,* 1985). In mung bean sprouts, the phenyl propanoid pathway was stimulated through Shikimate and Pentose Phosphate Pathways by the natural elicitors like peptides of fish protein hydrolysates (Randhir *et al.,* 2004). The authors showed that increased level of antioxidants was correlated with high Guaiacol peroxidase activity, indicating that the polymerizing phenols required during lignification with growth have antioxidant function. They hypothesized that enhanced mobilization of carbohydrates hastened phenol polymerization contributed to high antioxidant activity producing intermediary metabolites. Phenol polymerization also reduces the chance of interference of phenolic compounds with enzymatic reactions. Undoubtedly, as the molecular weight of phenolic compounds increases, its transport within the cell is considerably diminished. The two main types of phenolic polymers in plants are lignins and tannins. There are two main categories of tannin available in plant kingdom: hydrolysable and condensed tannins. Hydrolysable tannins are

conjugated form of gallic acid and glucose in different ratio. Condensed tannins i.e. Flavolans $(C_6\text{-}C_3\text{-}C_6)_n$ or proanthocyanidine by contrast, appear never to be associated with sugar, although there have been occasional reports of glucose being present (Harborne, 1998). It is already established that several tannins are potent inhibitors of gibberellin induced growth. The inhibition can be overcome with the additional GA_3 and there is no effect on endogenous growth. Thus tannins qualify as gibberellin antagonists (Corcoran *et al.* 1972). The biological activity of condensed tannins is limited by their high molecular weight and relative immobility. From the experiments with tannic acid [which is a mixture of gallic acid and various galloyl esters of glucose], it is observed that approximately 50% inhibition of amylase induction was observed when tannin and gibberellic acid tested ratio is 10:1, whereas this inhibition was 97% when the same ratio was 1000:1 (Figure 10.17). Clearly tannins particularly blocked the gibberellic acid mediated signal of amylase induction. As gibberellins appeared to play a dominant role in plant growth, more particularly in shoots, it is probable that tannins play a growth regulatory role by inhibiting growth caused by gibberellins in the plant.

The testa or the outer layer of the seed coat is assumed to play a vital role by controlling germination through dormancy imposition and by limiting the detrimental activity of physical and biological agents during seed storage (Mohamed-Yasieen *et al.* 1994, Weber *et al.* 1996). Most testa mutants showed reduced seed dormancy, as ascertained by a lower requirement for after-ripening and higher germination rate (Debeaujon *et al.* 2000). Over accumulation of the pigment in the seed coat is an obstacle to germination. From the vanillin or tetrazolium assays performed by authors it was concluded that the replacement of proanthocyanidine polymer by anthocyanin may lead to increased permeability of tetrazolium.

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