

Phenolic Signals and Their Perception Leads to *in Vitro* Shoot Induction in *Vigna radiata* Cotyledonary Explants

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ABSTRACT

The differential regeneration potential of two cotyledons (Cot and Cot E) of *Vigna radiata* seed during *in vitro* shoot differentiation is well established in this study. However, the two explant types influenced both the efficiency and mode of regeneration of each other when 8 Cot explants were placed at the periphery and 4 Cot E explants at the centre of Petri dishes containing shoot induction medium. Such a cross-talk was assumed to be mediated by exudates released from these explants in the agar medium. This led us to investigate the nature of the diffusing compound(s). Biochemical analyses revealed the phenolic nature of these diffusing metabolites. Experimentally it was demonstrated that these phenol-exudates had the potential to alter the mode of regeneration from callus-mediated to direct shoot formation in Cot explants and to enhance the shoot-promoting activity of Cot E explants. In contrast to the well defined role of phenolic compounds in plant defense, this study further showed the involvement of a HPLC purified phenolic compound, present in the exudates, in triggering *in vitro* shoot differentiation. Such a compound may be used to induce *in vitro* organogenesis, particularly in recalcitrant grain legumes.

Keywords: cotyledon, phenolics, shoot differentiation, *Vigna radiata*

Abbreviations: dpi, days post incubation; HPLC, high performance liquid chromatography; RT, retention time; SIM, shoot regeneration medium

INTRODUCTION

Mungbean (*Vigna radiata* (L.) R. Wilczek) is an important pulse crop, grown for its protein-rich edible seeds and sprouts in the tropical and subtropical region (Jaiwal *et al.* 2001; Tazeen and Mirza 2004). Even though it is well adapted to varied agro-climatic conditions, there is a need to develop *in vitro* protocol for the production of high yielding, disease resistant varieties. Most of the current transformation protocols invariably involve a tissue culture stage to recover plants (Hansen 1999). However, the *in vitro* regeneration of most grain legumes, including mungbean, is a challenging task due to their recalcitrant nature under *in vitro* condition.

In our laboratory, an *in vitro* regeneration protocol from cotyledonary explants of *V. radiata* has already been established (Pal *et al.* 1994; Chandra and Pal 1995; Chandra 1997). A differential regeneration potential was observed between the two types of cotyledons derived from the same seed in which one remains firmly attached to the embryonic axis (designated as Cot E) while the other (designated as Cot) is loosely attached to the embryonic axis. When grown individually in the same shoot induction medium (SIM), direct shoot regeneration was observed in the Cot E explants, while regeneration is callus-mediated and delayed in Cot explants (Chandra and Pal 1995). This unique phenomenon was further confirmed in subsequent studies (Das and Pal 2004; Das *et al.* 2006). It was also noted that when the two explant types were grown together, the mode of regeneration was dependant on their positional arrangement relative to each other (Das 2001). When 8 Cot explants were placed at the periphery and 4 Cot E explants at the centre of the Petri dishes containing SIM, direct shoot regeneration was observed in the Cot explants. On reversing the arrangement, i.e., Cot explants placed at the center and Cot E explants placed at the periphery of the Petri dish, the

regeneration efficiency of the Cot E explants reduced significantly and were callus mediated. This lead us to speculate that, when grown together, a crosstalk between the two types of explants determines the type of regeneration, either direct or callus mediated. This crosstalk in turn might be brought about by a signaling molecule released in the medium by either of the explants. Preliminary biochemical experiments indicated the compound to be phenolic in nature. Although phenolic compounds are broadly known to have deleterious effects on plant tissue culture, several reports indicate their growth promoting role in *in vitro* regeneration (Mato *et al.* 1988; Cvikrova *et al.* 2003; Ozygit *et al.* 2007; Reis *et al.* 2008).

In an effort to elucidate the nature of such a signaling compound if present, the current study was undertaken with both Cot E and Cot explants grown alongside in the same culture vessel and the culture media analyzed for chemical characterization of the exudates.

MATERIALS AND METHODS

Experimental materials

The de-embryonated cotyledons of an aromatic *Vigna radiata* Cv. B1, obtained from the Pulse and Oilseed Research Station, Behrampore, West-Bengal, India, were used as two separate explants, designated as Cot E and Cot as described by Chandra and Pal (1995). Briefly, the seeds were surface sterilized with 0.1% mercuric chloride for 2-3 min, thoroughly washed and aseptically imbibed in sterilized distilled water for 22 h. The seed coat was then removed carefully and the cotyledons were excised (Fig. 1).

Culture conditions

The explants were grown on Petri dishes (10 cm diameter × 1 cm height, Borosil) containing 15 ml Gamborg's B₅ nutrient medium

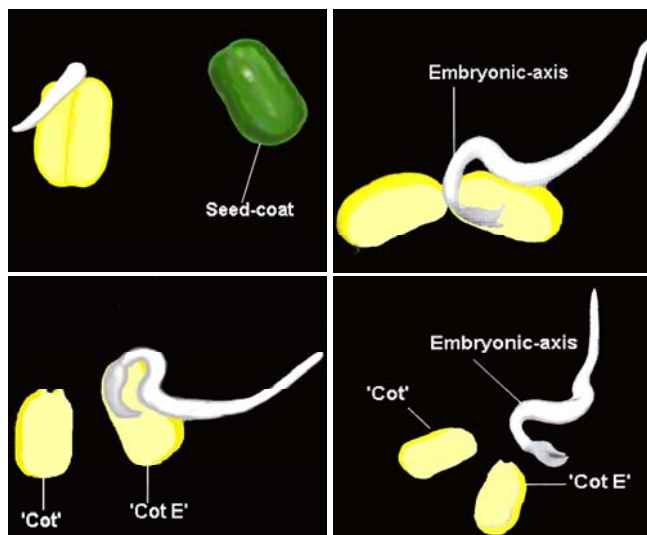


Fig. 1 Different steps for isolation of Cot and Cot E explants from the imbibed seed after removal of seed coat and finally excision of embryonic axis represented diagrammatically.

(Gamborg *et al.* 1968) supplemented with 15 μM N_6 -benzyl-aminopurine (Sigma, plant cell culture grade) and 0.8% agar (Himedia, Extrapure) as standardized earlier for shoot regeneration (Das and Pal 2004). The pH of the medium was adjusted to 5.7 prior to autoclaving. Cultures were maintained under a 16-h photoperiod at 75 $\text{mE m}^{-2} \text{s}^{-1}$ light intensity (white fluorescent bulbs, Philips, India) and incubated at $25 \pm 1^\circ\text{C}$.

Experimental design

Eight Cot explants were placed at the periphery and four Cot E explants were placed at the centre of the Petri dish containing SIM. Media were collected separately from 3 concentric regions of the Petri plates (zone 1 and 3 where Cot E and Cot were placed, respectively and zone 2 in between these two zones) after 6, 9 and 12 d post incubation (dpi) to isolate exudates from the explants. Medium without explants was used as control set. All the experiments were repeated at least three times.

Extraction and quantitative analysis of total phenolic compounds

The culture medium collected as above at 6, 9 and 12 dpi was extracted with 80% methanol (10 ml of methanol per mg medium) and centrifuged at 8000 rpm (4°C) for 10 min. The extraction process was repeated twice and the supernatant pooled and evaporated to dryness in a rota-evaporator (R-110 Buchi, Switzerland). The residue was dissolved in 300 μl of 70% aqueous acetone and diluted with 700 μl of ultrapure water. Total phenolics were estimated following the method of Reis *et al.* (2008) using Folin-Ciocalteu reagent and the absorbance measured at 760 nm in a spectrophotometer (Beckman coulter DU 520, Fullerton, CA, USA). All the samples were analysed in triplicate and phenolic contents of these samples were calibrated from the standard curve ($y = 0.0005x + 0.0123$, $r^2 = 0.9698$, where y is the optical density value of the sample, x is the concentration and r is regression coefficient; chlorogenic acid was used as standard). Computed values were represented as nanogram of phenolics secreted per gram of medium.

High performance liquid chromatography (HPLC) analysis of phenolic compounds

HPLC (Model UFLC 143, Shimadzu, Japan) was performed using RP-C18 column (Luna, 5u C18 (2) 100A; length, $250 \times 4.6 \mu\text{m}$; particle size, 4.98 μm). An isocratic solvent system comprising of 60% methanol and 40% trifluoro acetic acid (TFA, 0.01%) was used at 1 ml/min for 30 min for the separation of phenolic compounds (Chakraborty *et al.* 2007). The chromatograms were ana-

lyzed by LC Solution version 1.21 SP1 software (Shimadzu, Japan) and expressed in mg g^{-1} medium. HPLC purified phenolics were pooled and concentrated.

Effect of crude and HPLC purified phenolic compounds on *in vitro* regeneration

The effect of crude phenolics isolated as above and the HPLC purified phenolics on regeneration of the cotyledonary explants was checked. 100 μl crude ($1 \mu\text{g} \mu\text{l}^{-1}$) and 100 μl HPLC purified extracts per 15 ml medium were supplemented to SIM after filter sterilization by making a 0.5-cm bore well at the centre of each Petri dish. Two sets of experiments were performed for each extract, the first one containing 4 Cot explants placed concentrically outside the well and in the other 4 Cot E explants placed concentrically outside the well. Each experiment was repeated three times.

Data and statistical analyses

All the phenolic samples were analysed in triplicate and the mean data with standard errors were calculated.

RESULTS AND DISCUSSION

In vitro differentiation in Cot and Cot E explants

Swelling of the cotyledonary explants was observed within 3 days in culture. The growth of Cot E was faster than Cot explants. Direct shoot regeneration from the proximal end of the Cot E explants could be seen within 6 days. Regeneration in the Cot explants was preceded by callus formation and shoots were observed between 12 to 18 dpi.

Positional effect on regeneration percentage and mode of regeneration was apparent when 8 Cot explants were placed at the periphery of the Petri dish and 4 Cot E explants at the centre; direct shoot regeneration was achieved in the Cot explants (Fig. 2A). On the contrary, when Cot E explants were placed at the periphery of the Petri dish, the regeneration efficiency of Cot E was reduced significantly and callus formation preceded the shoot regeneration, as opposed to direct shoot regeneration (Table 1, Fig. 2B). Thus, a behavioural competency was observed when both the explants were cultured together *in vitro* and each type of explant influenced the regeneration potential of the other. We also observed a nutrient or metabolite pull towards the periphery where explants were more in number. The involvement of one or more secreted signaling molecule/s was postulated which on being secreted in the medium modulated the differentiation pattern of either of the explants. The differential response under these two experimental conditions suggests the possibility of two different types of signaling compounds, one exuded from Cot E pro-

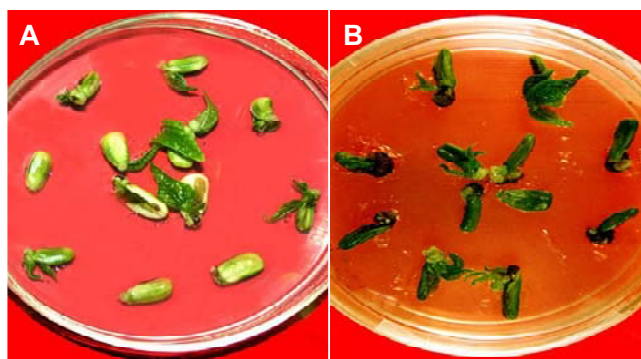


Fig. 2 Four Cot E explants placed at the centre of the Petri dish showing 75.0% direct shoot regeneration and 62.5% of the Cot explants, placed at the periphery, showing direct shoot regeneration at 9 dpi (A). Four Cot explants placed at the centre of the Petri dish showing 25.0% callus mediated shoot regeneration and 50.0% of the Cot E explants, placed at the periphery, showing direct shoot regeneration at 21 dpi (B).

Table 1 Regeneration frequency and mode of differentiation of Cot and Cot E explants when grown together for three weeks.

Explant type	Position of the explants	Total number of explants	Regeneration frequency (%)	Mode of regeneration
Cot	Peripheral	8 (5) ^a	62.5 (37.5) ^b	Direct regeneration
Cot E	Central	4 (3)	75 (100.0)	Direct regeneration
Cot E	Peripheral	8 (4-5)	> 50 (100.0)	Direct regeneration
Cot	Central	4 (1-2)	> 25 (37.5)	Callus-mediated regeneration

^a The number in parenthesis indicates the number of differentiating explants

^b Value within the parenthesis denotes the regeneration frequency obtained when the same explant type was grown separately, i.e. without any influence of other explant type

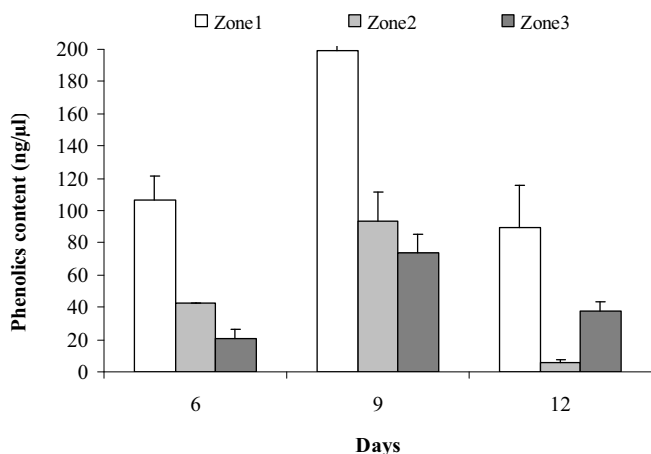


Fig. 3 Total phenolic contents (in ng µl⁻¹) isolated from the three concentric zones of agar medium at 6, 9 and 12 dpi.

moting regeneration; and the other exuded from Cot explants, suppressing regeneration. It has been previously elucidated by Das (2001) that the direction of nutrient pull was always in the direction where more explants were placed.

Nature of compounds secreted by Cot and Cot E explants into the medium during *in vitro* differentiation

Biochemical analysis of the medium on which the cotyledonary explants were regenerated and collected at 6, 9 and 12 dpi from the zones 1, 2 and 3, indicated the presence of phenolic compounds. Alkaloids, terpenoids and steroids were not detected (results not shown). Since initiation of shoot differentiation was observed at 6 dpi in the Cot E explants followed by shoot proliferation at 9 and 12 dpi, hence subsequent studies were carried out only at 9 and 12 dpi. Earlier, histological analysis of regeneration in cotyledonary explants revealed that shoot primordium initiated from Cot E explants on 4 dpi and leaf primordia appeared

on 6 dpi, followed by shoot growth on subsequent days (Das and Pal 2004). In the Cot explants shoot apical meristem and leaf primordia developing from callus tissue was observed earliest at 9 dpi.

A zone specific study of the phenolic exudates in the set with the Cot E explants placed in the center and Cot explants towards the periphery indicated that zone 1 contained the highest amount of the total phenolics followed by zone 2 and zone 3 at 6 dpi (Fig. 3). Same pattern of distribution was also observed at 9 dpi. While, at 12 dpi, zone 1 has the highest phenolic content, followed by zone 3 and zone 2 and the amount of exudates in three zones were lower than that accumulated at 9 dpi extracts. Hence, the present finding indicates a specific direction of metabolite diffusion from zone 1, where Cot E explants were placed and towards zone 3 where the Cot explants were placed.

Analysis of phenolic compounds through HPLC

HPLC data revealed a unique peak which resolved at 23.468 min RT (retention time) ($\lambda_{max} = 262$ nm) from zone 1, i.e., where the Cot E explants were placed. The compound was first detected in 9 dpi in all three zones (340, 360 and 600 ng g⁻¹ in zone 3, 2 and 1, respectively) with predominance in zone 1 (Fig. 4A-D). This is indicative of the release of the phenolic compound after the onset of regeneration in the Cot E explants which might play a role in shoot induction as previous histological studies have shown that shoot induction in Cot E explants grown in regeneration medium takes place within 4 dpi (Das and Pal 2004) and the excess metabolite is released into the medium afterwards.

Effect of phenolic compounds extracted from zone 1, 2 and 3 on regeneration of Cot explants

Direct shoot regeneration was observed in Cot explants when grown in the regeneration medium containing crude extracts from zone 1 and zone 2 placed in a well in the center of the Petri dish (Table 2). Regeneration frequency was same in the both the sets containing extracts collected at 9 and 12 dpi. However, the growth of the shoots was much better in sets containing extracts from zone 1 and 2 of

Table 2 Effect of phenolic extracts from nutrient medium at 9 and 12 dpi on regeneration frequency and mode of differentiation of Cot and Cot E explants when grown together.

Explant type	dpi	Extracts isolated from medium	Number of explants showing shoot differentiation	Regeneration Frequency (%)	Mode of differentiation
Cot	9	Control ^a	3 (8) ^b	37.5	Callus mediated regeneration
		Zone 1	3 (4)	75	Direct regeneration, faster shoot growth
		Zone 2	3 (4)	75	Direct regeneration, faster shoot growth
		Zone 3	1 (4)	25	Callus mediated regeneration
	12	Zone 1	3 (4)	75	Direct regeneration
		Zone 2	3 (4)	75	Direct regeneration
		Zone 3	0 (4)	0	Callus formation
Cot E	9	Control ^a	4 (4)	100	Direct regeneration
		Zone 1	4 (4)	100	Multiple shoot formation
		Zone 2	4 (4)	100	Direct regeneration
		Zone 3	3 (4)	75	Direct regeneration
	12	Zone 1	4 (4)	100	Multiple shoot formation
		Zone 2	4 (4)	100	Direct regeneration
		Zone 3	3 (4)	75	Direct regeneration

^a Without phenolic extract

^b The number in parenthesis indicates total number of explants

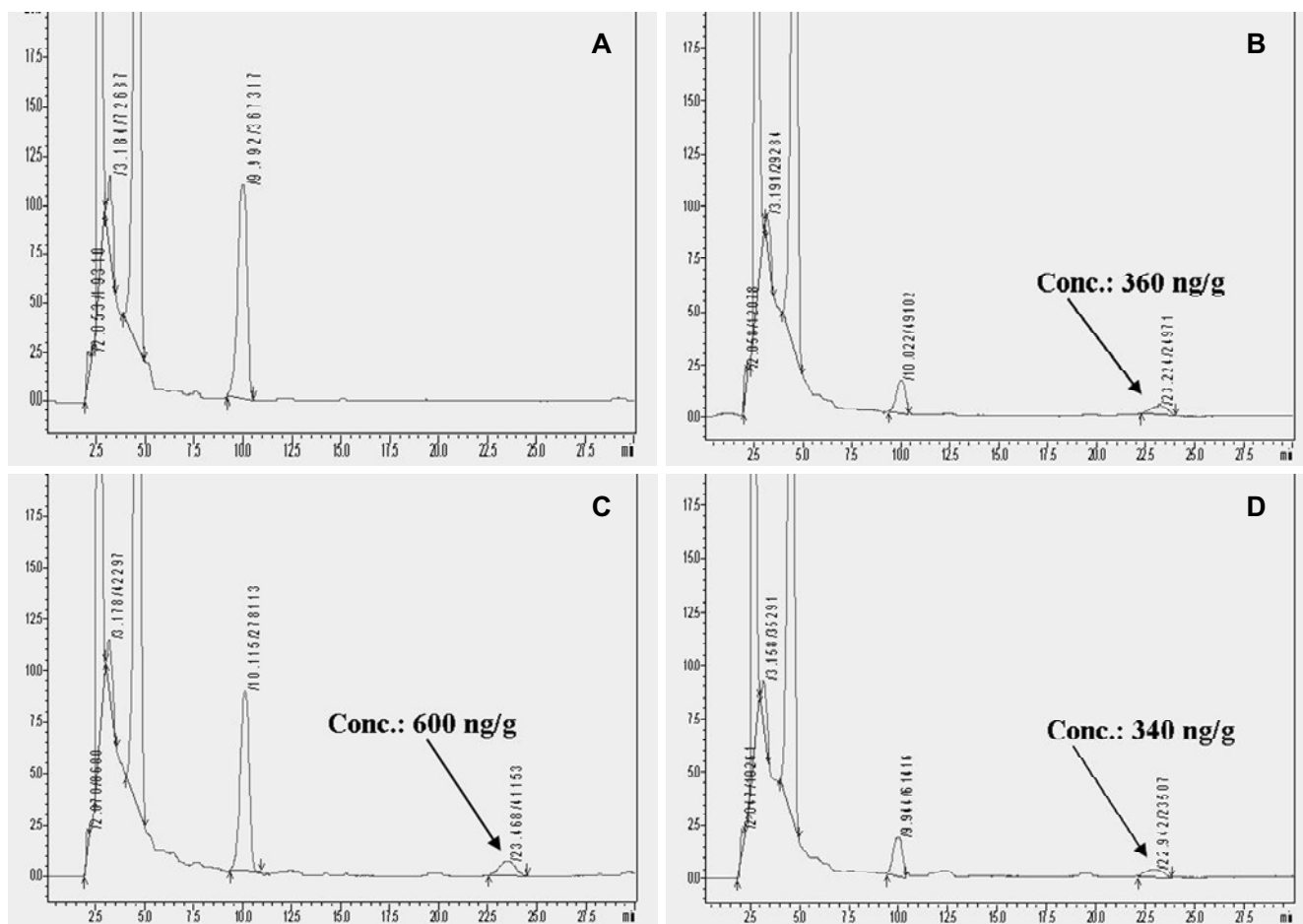


Fig. 4 HPLC diode array detector profiles of phenolic extracts from three different zones at 9 dpi. Control without any explants (A); zone 2 exudates (B); zone 1 exudates (C); zone 3 exudates (D).

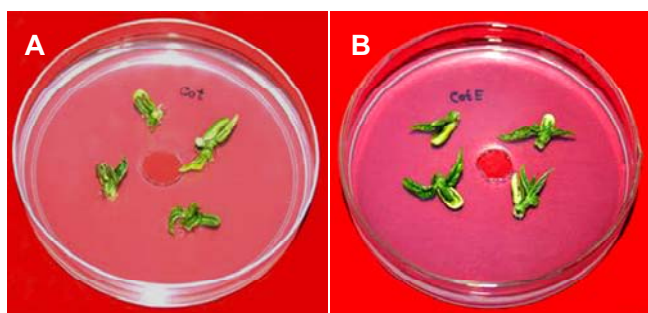


Fig. 5 Effect of a HPLC purified phenolic compound on shoot induction from Cot explants (A) and Cot E explants (B) at 12 dpi.

9 dpi. When zone 3 extracts obtained at 9 dpi were added to the well in the regeneration medium, callus formation was observed which was followed by shoot regeneration at 9 dpi but at a lower frequency. A probable explanation might be that the signaling compound secreted by Cot E explants is responsible for inducing direct shoot regeneration in Cot explants when added to the SIM. Further, the compound present in zone 3 is exuded by Cot explants and is antagonistic to shoot regeneration. Interestingly, when zone 1 exudates were supplemented in the medium containing only Cot E explants, multiple shoot regeneration was observed (data not shown) indicating the additive effect of the released phenolic compound(s).

Direct and 100% shoot regeneration from Cot explants was obtained when the HPLC purified phenolic compound was added to the well in the regeneration medium (Fig. 5A). Cent percent regeneration was also obtained in case of Cot E explants and the shoot growth was more prolific (Fig. 5B), which was probably due to the additive effect of the com-

pound.

Phenolic compounds and their glycosides are widespread in plants and have physiological and biochemical role primarily in plant defense (Dixon 2001), as signaling molecules (Mandal *et al.* 2010) and also in plant growth and development (Ozyigit *et al.* 2007). Phenolic concentration within the cell is regulated by several internal and external factors (Zaprometov 1989). Phenolic compounds are reported to be accumulated in the germinating seeds in *Brassica napus* concomitant with stages of embryo development and mobilization of storage material (Zobel *et al.* 1989).

The effectiveness of certain phenolic substances in enhancing shoot formation is well established in plant tissue cultures (George and Sherrington 1984 and references therein). Jones (1976) reported the positive influence of phloridzin and phloroglucinol, which promote the expansion of apple leaves and the rooting of apple shoots, respectively. Chirek (1990) demonstrated the presence of highest phenolic concentration in tobacco cell suspension culture before mitosis and the level reduced simultaneously with the onset of cell proliferation, perhaps by secreting the excess in the medium. Thus, it might be possible that certain phenolic compounds accumulating in the cotyledonary tissue during seed germination of *V. radiata*, were involved either directly to induce shoot differentiation during *in vitro* culture or acted as signaling molecules. The close association of the embryonic axis with the Cot E explants prior to excision could explain their better regeneration potential as these phenolic acids are invariably related to embryo maturity as reported in *B. napus* (Zobel *et al.* 1989). Reis *et al.* (2008) demonstrated that addition of phloroglucinol and caffeic acid at specific concentrations (197.5 and 140.0 μ M, respectively) in the regeneration medium increased the induction of somatic embryogenesis in *Feijoa sellowiana*. Ozyigit *et al.* (2007) are of the opinion that metabolized

phenols promote rapid cell division and synthesis of cell wall along with auxin metabolism in *Gossypium hirsutum* L. The present study advocates positive effect of phenolic compound at an optimal level on *in vitro* shoot induction in *V. radiata*. Lorenzo *et al.* (2001) experimentally demonstrated that there is a close relationship between phenolic production and shoot formation in *Saccharum officinales*, and the increased shoot induction is associated with an enhanced secretion of phenolics in the culture medium. This is in agreement with the *in vitro* response of *V. radiata* cotyledonary explants, where high phenolic content in the Cot E explants exuded in the medium can be correlated positively with direct and early shoot regeneration in comparison to Cot explants where regeneration is callus mediated and delayed.

Further investigations are underway to characterize the exact nature of this HPLC purified phenolic compound which may be used to overcome the recalcitrant nature of legumes *in vitro*, a formidable challenge in itself.

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