Journal of Pharmacognosy and Phytochemistry 2015; 4(4): 01-05

Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2015; 4(4): 01-05
Received: 05-09-2015
Accepted: 07-10-2015

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Rapid and highly competent shoot regeneration of Pigeon pea (Cajanus cajan) using variable explants by *in vitro* culture system

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Abstract
Pigeon pea is legume crop play a crucial role as source of dietary protein in diet, growing extensively in the rainfed and dryland spots of India and worldwide. Plant tissue regenerate through in-vitro system attempting organogenesis as well as embryogenesis pathway, which are in support of unfamiliar genes assimilation targeted for development of transgenic plants. Present study was undertaken to investigate the most appropriate explant type in Pigeon pea regeneration by virtue of *in vitro* culture system. Genotype Durga (NTL-30) was breed and used as principal material for regeneration studies. Explants isolation from *in vitro* elevated germinating 6-8 days old seedlings were used for embryonic axis and cotyledonary node, whereas isolation of Scutellum (IZE) explants from overnight imbibed seed. Isolated explants were cultured on Murashige and Skoog medium supplemented with zeatine (0.572, 1.35, 1.47, 2.32 µM) and kinetin (0.46, 0.93, 1.39 & 1.86 µM) as plant growth regulator for regeneration. Additionally medium incorporated with 2.94 µM of silver nitrate to accelerate growth. Successive to regeneration, shoots bud originated from embryonic axis, cotyledonary node and Scutellum were rapidly elongated on MS medium included 0.50 µM gibberlic acid and further rooted on M.S. medium cantoning idol- acetic acid 0.57 µM which notice 95% efficient root induction. The methodology reported here is very simple, skilled and reproducible, can be employing diagonally to corresponding genotypes of pigeon pea, thus can be practically useful for the establishment of transgenic plants.

Keywords: Cajanus cajan, shoot regeneration, *in vitro* culture system, Scutellum-Immature zygotic embryo(IZE).

1. Introduction
Pigeon pea widely well-known as red gram also locally "tura" in India, it is most significant food legume having crucial major grain legumes of the semi-arid tropics. It is grown commercially throughout the globe and cultivated in about fifty countries of Asia, Africa, and America for food, fodder, fuel, soil conservation, and green manure [Krishna G *et al*. 2011] [1]. The productivity of pigeon pea are introverted by numerous diseases, including sterility mosaic, fusarium wilt, Phytophthora blight, Alternaria blight and major insect pest *Helicoverpa armigera* [Reddy MV *et al*. 1990] [2]. Biotechnological application such as genetic transformation headed for superior pest resistance put forward opportunities for rapid improvement of pigeon pea. However the accessibility of an *in vitro* regeneration method is a prerequisite for effective plant regeneration. Regeneration through *in vitro* culture for exploitation of plant cell totipotency shows organogenesis and regeneration of shoot buds from various explants type of pigeon pea has been reported earlier by [Shrinivasan T *et.al* 2004, Geetha N *et al*. 1998 [4], Shiva PN *et al*. 1994] [3, 4, 5]. Regeneration of pigeon pea via organogenesis has been reported with pre-existing meristem like apical meristem [Cheema HK *et al*. 1991] [6]. Cotyledonary node [Geetha N *et al*. 1998] [4]. Embryonic axis [Krishna G *et al*. 2010] [7].
The above published literatures suggest that addition of cytokinines in regeneration medium could be the underpinning for pigeon pea shoot bud discrimination [Dayal S *et al*. 2003] [8]. Silver nitrate as an additive in medium promote plant growth regulation and morphogenesis in recalcitrant crop such as pigeon pea by means of participating silver ions in the form of nitrates as primary role and influencing somatic embryogenesis. Efficient shoot and root development which is the primary prerequisites for successful genetic transformation, reported earliear [Bais HP et *al*. 2000] [9]. Hence forth the aim of the present research to fill up this unfilledness of shoots regeneration, proliferation and *in vitro* plantlets development. Pigeon pea regeneration

through *in vitro* by addition of hormonal combination reported is rapid, Effortless and efficient for regeneration of cotyledornary node, embryonic axis and immature zygotic embryo (Scutellum) explants.

2. Material and Method

2.1. Plant material

Healthy seeds of Pigeon pea Durga (NTL-30) were obtained from Nirmal Seeds Pvt. Ltd., Pachora, Jalgaon, (M.S.) India. Mature uniform seeds were surface sterilized by rinsing 2-3 times with distilled water, followed by treatment with solution of concentrated H_2SO_4 (v/v) for two minute in favour of slacken the seed coat, seeds were then rinsed thoroughly with sterile distilled water. The seed were consequently transferred in 2% bavistine for 60 minute incubation period; the seeds were thereafter rinsed with sterile distilled water. Subsequently seeds were treated with 0.01% $HgCl_2$ for 30 minute and wash severely again with distilled water. Seeds were air dried and germinated on MS half strength basal medium supplemented with BAP 8.90 µM. The pH of the medium was adjusted 5.8 and sterilized at 121 ^{0}C for 15 minute. Seeds were incubated at room temperature (27 ± 1 ºC) for 16 hours photo-period.

2.2. Explants preparation

Explants were prepared from six to eight days old in-vitro grown germinating seedlings. Embryonal axis explants Isolation from basal swollen region of the excised seedling by using germinating seedling. Cotyledonary node isolated from apical region of seedling by incising both cotyledonary leaf from base and apical primary leaf (Figure- 1).

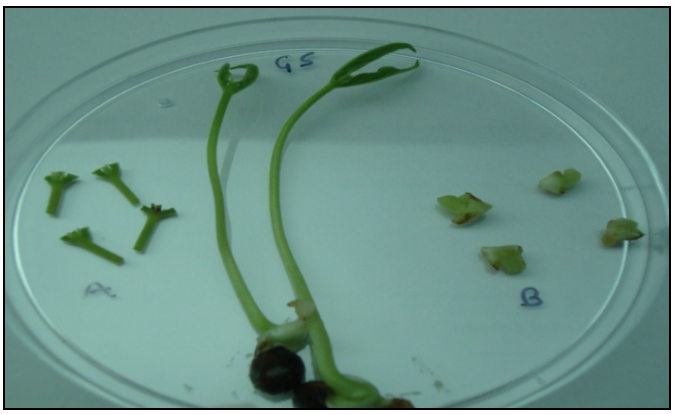

Fig 1: Cotyledonary node exlants (a), embryonic axis explants (b) from germinating seedlings (GS).

Scutellum/ immature zygotic embryo (IZE) explant were isolated using overnight imbibed surface sterilized healthy seeds dried using sterile filter paper, seed testa were removed and exposing the zygotic immature embryo and cotyledons. The cotyledons were separated by slight incision from central axis forming immature zygotic embryo. Nourishing tissue with zygotic embryo was used as the starting explants material for regeneration. Isolated explants were transferred in MS liquid medium without drying. (Figure- 2).

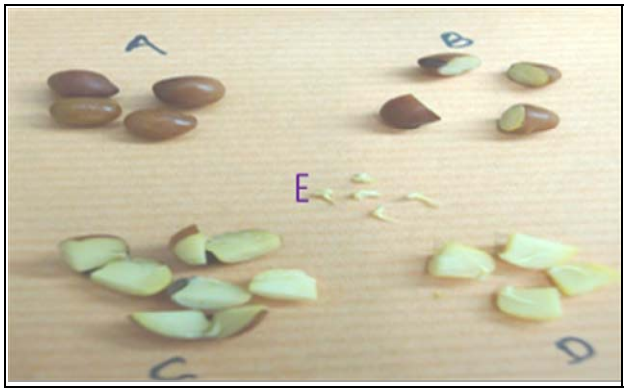

Fig 2: Scutellum explants preparation stages A) whole seed B) Cotyledon excise from axis, C&D) Scutellum with attached cotyledon E) Scutellum

2.3. Shoot bud Regeneration

In order to regenerate shoots of Pigeon pea cultivars Durga (NTL-30) through organogenesis using embryonic axis, cotyledonary node and sutellum, explants were cultured on shoot induction MS medium supplemented with 58.8 µM $AgNO_3$ [Ignacimuthu S *et al*. 1999] [10]. The various concentration of plant growth regulator zeatin 0.57, 1.35, 1.47, 2.32 µM and kinetin 0.46, 0.93, 1.39, 1.86 µM in combination as well as solitary were tried. The optimal concentration for shoot regeneration integrates with zeatin 1.35 µM and kinetin 0.93 µMat highest efficiency. The pH of the medium was adjusted to 5.8 before autoclaving and medium was solidified by 0.8% Agar-agar. Explants were incubated at 25 ± 2 °C under l6 hour's photo-period for two week. Shoot bud were elongated on elongation medium containing GA_3 0.50 µM. After incubation, the responses of shoots elongation were recorded and the elongated shoots were transferd on root induction medium supplemented with IAA-0.57 µM. The abundant grown rooted plants were ultimately planted in coco peat filled in small pots supplemented with water and 65 % humidity in growth room conditions for 15 -20 days, thereafter acclimatize in glass house for further adaptation.

3. Results and Discussion

3.1. Seeds germination

The seed germination on half strength MS medium having 8.90 µM BAP were shown enhancing rate of germination reach up to 95 percent. Benzylaminopurine prominently facilitate swollen basal section of seedling and developing embryonic axis while absence of BAP establishment deformation of embryonic axis explants and cotyledonary node at apical region. Utilization of concentrated sulphuric acid during germination slackens the seed coat and condenses the elevation of infectivity parallel work previously reported [Lawrence PK *et al*. 2001] [11].

3.2. Shoot Regeneration

In the present study zeatin and kinetin combination was found to be appropriate for shoot induction and proliferation. Frequency of shoot regeneration was found to be highest in

embryonic axis, followed by cotyledonary node and Scutellum explants (Table 1). Accumulation of $AgNO_3$ 2.35 µM, 2.94 µM, 4.70 µM, 5.88 µM were attempt to examine the effect on shoot regeneration response (Table 2) and it was found that optimum 2.94 µM support the maximum multiple shoot development (Fig 3a, Fig3b,Fig 3c)

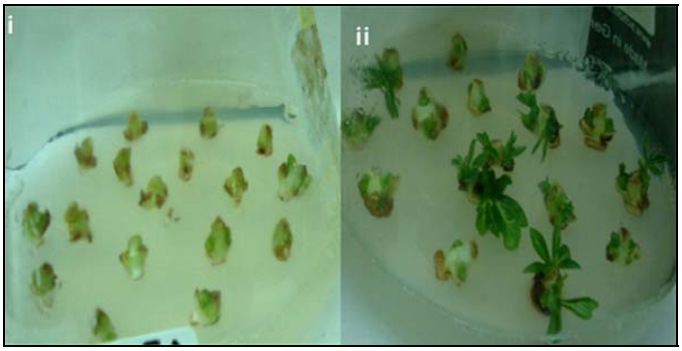

Fig 3a: Embryonal axis explants (i) shoot development (ii).

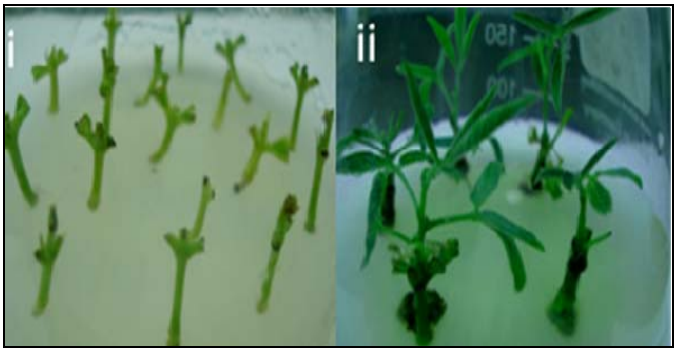

Fig 3b: Cotyledonary node explants (i) shoot development (ii).

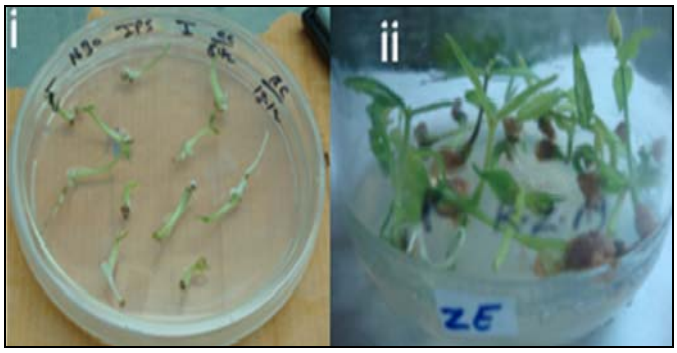

Fig 3c: Imature zygotic embryo (Scutellum) explants (i) shoot development (ii).

Table 1: Effect of explants type on shoot induction through *In-vitro* culture system of pigeon pea

Explants type	No. of explants cultured	No. of explants producing shoot bud	Frequency of shoot bud induction (%)
Cotyledonary node	50	40	80
Embryonic axis	50	48	96
Scutellum	50	37	74

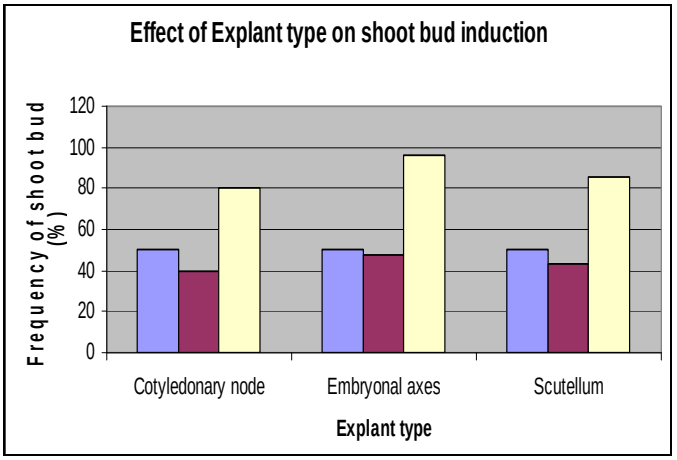

Fig 4: Effect of explants type on shoot but induction in pigeon pea

Embryonic axis, cotyledonary node and Scutellum explants was cultured on MS medium (MSZKb) supplemented with zeatin 1.35 µM and kinetin 0.93 µM alongside 2.94 µM $AgNO_3$ shown 96% shoot regeneration with shoot length of 2.0-4.8 cm recorded (Table 2). Alone zeatine as well as kinetin unable to influence efficient shoot formation, however in combination it facilitate highest efficiency headed for shoots proliferation passing through silver nitrate. The intermission of shoot elongation was overcome by shifting to a shoot elongation medium contain GA_3 0.50 µM shows distinctly lengthen shoots (Table 3). Elongation medium proficient for plantlets enlargement with 89 percent and shoot length of 4.10- 4.8 cm was reported (Figure 5). The maximum frequency of shoot formation was 74 to 96 percent on regeneration medium (Table-1). The lowest regeneration frequency has shown 48 to 60 percent, the regeneration frequency superior in the concentration of lesser altitude of cytokinine is optimal for shoot bud formation. Higher altitude concentration of cytokinine above 3 µM shows shoot frequency decline drastically.

Table 2: Effect of varied concentration of Zeatin and kinetin on shoot bud induction and contributory role of AgNO3 from embryonal axis, Cotyledonary node and Scutellum explants of pigeon pea

Medium	Growth regulator (µM)			No. of explants cultured	Explants producing shoot	Explants forming Shoots (%)	Shoot length in cm
	Zeatin	Kinetin	$AgNO_3$				
MSZa	0.572	-	2.35	100	60	60	2.2
MSZb	1.350	-	2.94	100	50	50	2.3
MSZc	1.472	-	4.7	100	48	48	2.5
MSZd	2.320	-	5.88	100	52	52	2.0
MSZKa	0.572	0.46	2.35	100	92	92	4.2
MSZKb	1.350	0.93	2.94	100	96	96	4.8
MSZKc	1.472	1.39	4.70	100	88	88	4.3
MSZKd	2.320	1.86	5.88	100	80	80	4.1
MSKa	-	0.46	2.35	100	70	70	3.1
MSKb	-	0.93	2.94	100	68	68	3.0
MSKc	-	1.39	4.70	100	66	66	3.2
MSKd	-	1.86	5.88	100	76	76	3.2

3.3. Root development and transplantation

Elongated Shoots 3.5 to 4.0 cm extended was relocating to root induction medium supplemented with indol-3 acetic acid 0.57 µM (Table 3). Regenerated plantlets with sprouted roots (figure. 5) were transfer to pot filled with coco peat in growth room conditions at 65% humidity for three week subsequently acclimatisation in glass house for further adaptation.

Table3: Effect of PGR on shoot elongation and root induction of proliferated shoot

conc. of GA3 (µM)	% of shoot elongation	Average shoot length(cm)	conc. of IAA (µM)	% of root induction	length of roots (cm)
0.25	78	4.2	0.28	69.5	3.5 +1
0.50	89	4.8	0.57	95.0	5.0 +1
0.75	45	4.3	0.85	80.0	4.0+1
1.25	40	4.1	1.14	75.0	2.5+1

Embryonic axis, cotyledonary node and Scutellum explants regeneration is focussed, extremely simple and less time consuming development towards huge number of plantlets. Regenerated Plants produced are true to phenotype with insignificant rate of somaclonal variation and chromosomal abnormalities.

Fig 5: Shoot elongation and root development

Embryonic axis explants reveal that profuse shooting at elevated rate and more appropriate since meristematic cells are premeditated reported in *Vigna mungo* [Ignacimuthu S *et al*. 1999] [10]. Hence employing implement to achieve higher rate regeneration and same results were obtained. Present experiment concludes embryonic axis produced a bunch of shoot bud hence it is the most amiable explants for regeneration (96%). Cotyledonary node is the second prior explant for producing mass of multiple shoot at the auxiliary bud region therefore considered as more appropriate for regeneration proven by Shiv prakash 1994 [5]. Considering pigeon pea is recalcitrant nature both cotyleonary node and embryonic axis achieved contemporary 80% regeneration response.

Immature zygotic embryo (IZE) tenure as Scutellum is the third preferably explants for generating high frequency for shoot regeneration induced by exogenous auxin to proliferate by mean of somatic embryogenesis reported in maize [Frame BR *et al*. 2002] [12]., on the contrary allowing for fruitfully producing maximum plantlets and observed 74% regeneration during contemporary observation.

Plants behavioural study for regulating morphogenesis usually by hormonal balance which is a key factor *in vitro* culture system .The optimum hormones combination ratio facilitate to influence plant enzymatic reactions resulting of plant growth and important to the particular effect on growth and morphological amendment proven [Satyavathi VV *et al*. 2002] [13]. Existing result also indicated that the regeneration and elongation of shoot was promoted by equilibrium of cytokinin. In the contemporary study hormone combination of zeatin 1.35 µM and kinetin 0.93 µM in MS medium generate better for shoot regeneration (96%). The best possible level of hormonal combination zeatin and kinetin support the highest number of shoot bud proliferation, no report has been published yet with these combinations. Elongation of regenerated shoot found 4.80 cm in MS medium containing 0.50 µM GA_3 and revealed 85 percent enlargement. Establishment of roots towards elongated shoots is essential for successful plantlets in the soil. As per the requirement of plantlets adaptation in soil with profuse rooting cotyledonary node and embryonic axis show highest rootingiu frequency (95.0%) in media containing 0.50 µM of IAA.

Additionally to the medium silver nitrate incorporate as well considering adverse effect on shoot induction *in vitro* by recognized to promote multiple shoot formation and adsorbing phenolic compound in the medium to inhibit the division of cell in different plants. *In vitro* shoot formation was improved by incorporating silver nitrate in the culture medium reported [Ganesh *et al*.1996] [14]. Low concentration $AgNO_3$ was found to cause delayed senescence resulting in improved growth of the proliferated shoots. Silver nitrate was found to be beneficial in the regeneration peanut [Pestana MC *et al*. 1999] [15]. Cowpea [Brar MS *et al*.1999] [16]. Ongoing results show incorporation of 2.94 µM $AgNO_3$ significantly enhances shoot proliferation.

4. Conclusion

The novelty of the cited research shows that combination of cytokinin i.e. zeatin and kinetin responded vigorously to shoot induction for cotyledornary node, embryonic axis and Scutellum explants, which was earlier reported incredibly time consuming through a variety of auxin and cytokinin combination. The shoot proliferation with silver nitrate as additives expose at superior velocity. The above three mention explants work out on single hormonal combination for regeneration and no reports were found with such single-handedly combination . Root induction was produced within little time period of which is prerequisite for hardening. This was the first report to employ silver nitrate along with cytokinin combination which facilitates regeneration with elevated velocity within stipulated time. Current protocol is based on regeneration of shoot from the embryonic axis, cotyledornary node and Scutellum with high competency and is highly appropriate for genetic transformation.

5. Reference

1. Krishna G, Reddy PS, Ramteke PW, Pogiri R, Sayed SS, Rana D *et al*. *In vitro* regeneration through organogenesis

and somatic embryogenesis n pigeon pea (Cajanus Cajan (L.). Physiol Mol Biol Plants 2011; 17(4):375-385.
2. Reddy MV, Sharma SB, Nene YL. Pigeon pea: disease management. In: Nene YL, Hall SD, Sheila VK (Eds) the pigeonpea. CAB, Wallingford, UK1990; 303-347
3. Shrinivasan T, Verma V, Kirti P. Efficient shoot regeneration in pigeon pea, Cajanus cajan (L) Millisp.using seedlingpetioles. Current science 2004; 86:1.
4. Geetha N, Venkatachalam P, Prakash V, Lakshmi Sita G. High frequency induction of multiple shoots and plant regeneration from seedling explants of pigeon pea (Cajanus cajan L.). Curr Sci 1998; 75:1036-1041.
5. Shiva Prakash N, Pental D, Bhalla SN. Regeneration of pigeon pea (Cajanus cajan) from cotyledonary node via multiple shoot formation, Plant Cell Rep1994; 13:623-627.
6. Cheema HK, Bawa J. Clonal multiplication via multiple shoots in some legumes (Vigna unguiculata and Cajanus Cajan). Acta Hortic1991; 289:93-94.
7. Krishna G, Reddy PS, Ramteke PW, Bhattacharya PS. Progress of tissue culture and genetic transformation research in pigeon pea [Cajanus Cajan (L.) Millsp.]. Plant Cell Rep 2010; 29:1079-1095.
8. Dayal S, Layanya M, Devi P, Sharma KK. An efficient protocol for shoot regeneration and genetic transformation using leaf as explants. Plant Cell Rep 2003; 21:1072-1079.
9. Bais HP, Sudha GS, Ravishankar GA. Putrescine and AgNO3 influences shoot multiplication *In vitro* flowering and endogenous titres of polyamines in Chichorium intybus L cv Lucknow Local. Journal Plant Growth Regulation. 2000; 19(2):238-248.
10. Ignacimuthu S, Franklin G. Regeneration of plantlets from cotyledon and embryonal axis explant of Vigna mungo L. Hepper Plant Cell, Tissue and Organ Culture 1999; 55:75-78.
11. Lawrence PK, Koundal KR. Agrobacterium tumefaciens-mediated transformation of pigeon pea (Cajanuscajan L. Millsp.) and molecular analysis of regenerated plants. Current science 2001; 80:11.
12. Frame BR, Shou H, Chikwamba RK, Zhang Z, Xiang C, Fonger TM *et al*. Agrobacterium tumefaciens-mediated transformation of maize embryos using a standard binary vector system. Plant Physiol 2002; 129:13-22.
13. Satyavathi VV, Prasad V, khandelwal A, Shaila MS, Lakshmi Sita G. Expression of hemagglutinin protein of Rinderpest virus in transgenic pigeon pea [Cajanus cajan (L.) Millsp.] Plants. Plant Cell Rep 2002; 21:651-658.
14. Ganesh SD, Sreenath HL. Silver nitrate enhanced shoot development in cultured apical shoot buds of Coffea arabica Cv Cauvery (S4347). Journal of Plantation Crops. 1996; 24:577-580.
15. Pestana MC, Lacorte C, VG de Freitas, Dulce De Oliveira, Mansur E. *In vitro* Regeneration of Peanut (Arachis Hypogaea L.) Through Organogenesis: Effect of Culture Temperature and Silver Nitrate. *In vitro* Cellular & Developmental Biology-plant 1999; 35(3):214-216.
16. Brar MS, Moore MJ, Al-Khayri JM, Morelock TE, Anderson EJ. Ethylene inhibitors promote *in vitro* regeneration of cowpea (Vigna unguiculata L.). *In vitro* Cell. Dev. Biol. Plant 1999; 35:222-225.