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Direct differentiation of shoot buds from leaf explants of *Cajanus cajan* L.

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Abstract

A protocol was developed for direct differentiation of multiple shoot buds from leaf explants of *Cajanus cajan*. In a modified Murashige and Skoog's medium supplemented with 2.22 μM benzyladenine (BA), 0.57 μM indole-3-acetic acid (IAA) and 41 μM adenine sulphate (AdS), the segments of basal halves of the first two leaves of a young seedling incubated on filter paper bridges in liquid medium took 20 - 25 d to differentiate shoot buds. The explants after transfer to solidified medium, with lower concentration of BA (0.22 μM) resulted in fast growing healthy shoots. The developed shoots (measuring *ca.* 3 cm) were rooted in a medium supplemented with 1.42 μM IAA. They were subsequently grown in pots with soil with more than 80 % transplantation success.

Additional k*ey words*: agar medium, liquid medium, pigeon pea, plant regeneration, histology.

Introduction

Cajanus cajan (pigeon pea), a major grain legume for subsistence in tropics, has been subjected to *in vitro* studies not only for assessing its amenability for regeneration, but to establish a practical system for genetic transformation (George and Eapen 1994, Prakash *et al.* 1994). *C. cajan* have proved recalcitrant to regenerate by Polanco and Ruiz (1997). For plant genetic transformation the co-culture of *Agrobacterium tumefaciens* and leaf segments (disc) is a preferred method (Horsch *et al.* 1985). Besides, leaf segments being used as explants for genetic tranformation, the direct regeneration without a callus phase is a pre-requisite least genetic imbalances should be mixed up with the genetically transformed traits. However, despite several attempts and a number of reports, it has not been possible to achieve direct regeneration from leaf segments of *C. cajan*, where most of the reports are on callus tissue (Kumar and Reddy 1983, Eapen and George 1993, George and Eapen 1994, Patel *et al.* 1994, Sreenivasu *et al.* 1998). In certain other cases where it is reported to be a direct embryogenesis or shoot bud organogenesis, it has been with a fully differentiated storage organ, *i.e.* cotyledon (Patel *et al.* 1994, Sudarsana Rao *et al.* 2001). But cotyledons had varied ploidy levels and frequently consisted of genetic chimeras (Smith 1971, Scharpe and Van Parijs 1973, Nagl 1978). Also, the system proposed in a paper on differentiation of shoot buds from cotyledonary nodes of *C. cajan* can hardly be a match to a leaf disc method for bacterium mediated genetic transformation studies, both for the ease of the system and the number of cells of explants available for the bacterium to invade (Prakash *et al* 1994, Geetha *et al.* 1998). Furthermore, stem and root explants pose difficulty with Ti plasmids bereft of tumor inducing genes (Horsch *et al.* 1985). We now report, for the first time, direct *de novo* differentiation of multiple shoot buds from leaf lamina explants of *C. cajan*, their development into complete plants *in vitro* and their transplantation to soil.

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Abbreviations: AdS - adenine sulphate; BA - benzyladenine; CCC - 2-chloroethyltrimethyl ammonium chloride; IAA - indole-3-acetic acid; 2iP - 2 isopentenyladenine, Kn - kinetin; MS - Murashige and Skoog's (1962) medium; SH - Schenk and Hildebrandt's (1972) medium; FPB - filter paper bridge.

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Materials and methods

Seeds of *Cajanas cajan* L. cv. Bahar having a better taste than other cultivars available in market were surface-sterilized with 0.1 % $HgCl_2$ (8 - 10 min) after pre-treating them with wetting agents. Thoroughly washed seeds were left overnight in sterile water to be germinated in White's (1943) medium solidified with 0.7 % agar. The pH of all media was adjusted to 5.8 and they were sterilized by autoclaving at 1.08 kg cm^{-2} for 15 min. Cultures were incubated under 14-h photoperiod with irradiance of 36 μmol $m^{-2}s^{-1}$ and temperature 27 ± 1 °C.

Seven to 10-d old *in vitro*-grown seedlings, which had produced the first two fully expanded simple leaves (other than the cotyledons) and another well-developed trifoliate leaf besides the shoot tip were used as the source for leaf explants. Ten replicate cultures were observed for each treatment. Each culture having 3 segments of about 5-mm-wide transverse sections of a leaf cut from the first two leaves and also from the leaflets of the third leaf were used as explants. All the segments were inoculated with their abaxial face onto the solidified nutrient medium as well as onto the filter paper bridges, using liquid nutrient medium. Modified media of Murashige and Skoog (1962) or Schenk and Hildebrandt (1972) were used as the basal media. In order to derive a most suitable basal medium, the various concentrations of NH_4NO_3 (6.25 - 25 mM), KNO_3 (4.95 - 198 mM), and $CaCl_2 . 2\ H_2O$ (0.68 - 3.4 mM), Na_2SO_4 (0.35 - 1.41 mM), L-glutamine (34 - 340 μM), L-arginine-HCl (24 - 95 μM) and L-tyrosine (28 - 280 μM) were used. Composition of the most effective basal medium (BM1) was: 12.5 mM NH_4NO_3, 9.9 mM KNO_3, 1.4 mM $CaCl_2 . 2\ H_2O$, 0.7 mM Na_2SO_4, 171 μM L-glutamine, 48 μM L-arginine and 28 μM L-tyrosine. For further development of shoot buds differentiated in leaf explants, the basal medium was further supplemented with 758 μM $(NH_4)_2SO_4$. With the finally selected basal medium, morphogenic effects of different cytokinins, like Kn (0.04 - 4.6 μM), BA (0.04 - 4.4 μM) or 2iP (0.03 - 3 μM), and AdS (14 - 109 μM), used in the presence of IAA (0.57 μM), were seen for induction of differentiation of shoot buds in leaf explants as well as for promoting growth of regenerated shoots. The developed shoots, measuring *ca.* 3 cm in length were excised and subjected to *in vitro* rooting in the medium supplemented with IAA (0.57 - 2.85 μM). The plantlets were transplanted to pots with sterilized mixture (soil and leaf mould 1:1) for their further growth in the glasshouse.

Leaf explants with differentiated shoot buds at various developmental stages were processed for histological study with microtome sections using haematoxylin and safranin as differentiating stains (Johansen 1940).

Results and discussion

Shoot bud differentiation took place in the explants taken from the basal and middle portions of lamina of the first two leaves and not in those excised from its tip portion, while the explants from the third leaf, only expanded and became variously undulated without any regenerant formation. It is interesting that the innate regeneration (caulogenic) potentiality was restricted only to the first two leaves and absent in the third (trifoliate) leaf. The involvement of juvenile factor, reflected in the simple nature of first two leaves in a plant, which otherwise has adult trifoliate leaves, appears to be the main cause of such a differential response of leaf explants. It indicates a closer affinity of first two leaves with the cotyledons, which have been found most regenerative in *C. cajan* (Kumar and Reddy 1983, George and Eapen 1994, Patel *et al.* 1994).

Among three different cytokinins tested only BA was effective in inducing shoot bud differentiation, while Kn induced callusing and 2iP caused browning of the cultures (Table 1). Liquid medium with filter paper bridge was essential to obtain shoot bud differentiation without any intervening callusing (Fig. 1*A*) even in BA treatment, while in the corresponding cultures on nutrient agar, only an excessive callusing of explants was obtained in BM1 supplemented with 2.22 μM BA, 0.57 μM IAA and 41 μM AdS. It is difficult to explain that addition of agar to the medium changed the efficacy of BA. In the present study in solidified medium, BA presence results into excessive cell division resulting into callusing of explants, while in liquid medium it tends to differentiate shoot buds directly. However, it is a general observation that the first step preceding organized differentiation is slowing down the cell division (Chaturvedi and Mitra 1975).

Table 1. Effects of different concentrations of BA on shoot bud differentiation from leaf explants in *C. cajan* after culture period of 45 d. Means of 10 replicate cultures ± SE.

BA [μM]	Response [%]	Number of buds	Callusing
0.04	40	15 ± 1.3	-
2.22	90	45 ± 3.4	-
4.40	80	20 ± 2.3	+

Meristematic activity, particularly at the cut edge of the explants proximal to petiole and near the midvein as also the other prominent veins, ensued after about 10 d of

Table 2. Shoot bud differentiation in the medium BM1 with 2.22 μM BA + 0.57 μM IAA + 41μM AdS in liquid medium on FPB in leaf explants of *C. cajan* after culture period of 45 d. Means of 10 replicate cultures ± SE.

Leaf segment	Near midvein number of buds	response [%]	Near lateral vein number of buds	response [%]	Interveinal portion number of buds	Number of buds response [%]
Lower petiolear	18 ± 1.52	90	6 ± 0.51	80	4 ± 0.22	70
Middle portion	12 ± 0.93	80	2 ± 0.02	60	1 ± 0.00	50
Tip portion	2 ± 0.03	20		0		0

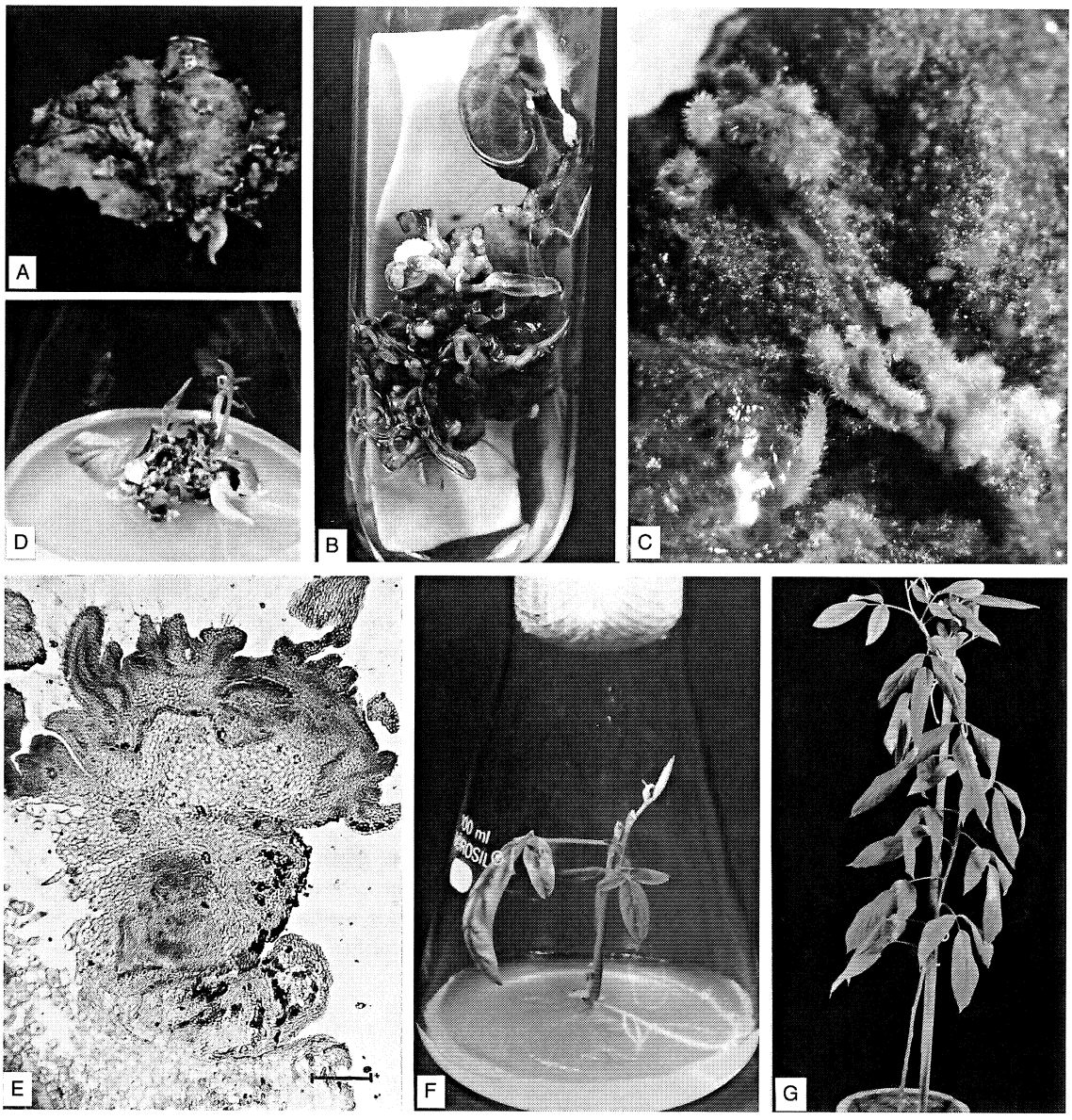

Fig. 1. Cultures of *Cajanus cajan*: *A* - differentiation of multiple shoot buds directly from a segment of leaf lamina; *B* - differentiation of multiple shoot buds from a leaf explant cultured on a filter paper bridge with liquid nutrient medium; *C* - a magnified view of just differentiated shoot buds along the mid rib as also in the interveinal portion without any callus formation; *D* - development of healthy shoots from shoot buds differentiated in leaf explant, while several new shoot buds are differentiated at the base; *E* - histogenesis of shoot buds differentiated directly from leaf laminar tissue without intervening callus as seen in a vertical section (*bar* = 200 μm); *F* - a rooted shoot; *G* - leaf tissue-regenerated plant of *C. cajan* grown in potted soil 2 months after transplantation.

incubation (Fig. 1*A*). After another 10 - 15 d, shoot buds were apparent in these regions of meristematic activity as well as occasionally in the interveinal portion of intact leaf lamina, while the explants expanded to about 2 - 3 times (Table 2, Fig. 1*B*). Differentiation of shoot buds, in groups, from the laminar tissue of the leaf explant with virtually no interveining callusing is seen (Fig. 1*C,E*). The shoot buds were restricted in their further growth under the same conditions, in which they had differentiated. On the other hand, portions of explants with differentiated shoot buds when transferred to modified BM1 having an additional supplement of 758 μM $(NH_4)_2SO_4$, increased concentration of AdS (68 μM) and decreased concentration of BA (0.22 μM), the shoot buds developed rapidly into leafy shoots while several new shoot buds continued to differentiate (Fig. 1*D*). A maximum number of 28 shoot buds differentiated from the lower petiolar portion, whereas 45 shoot buds differentiated from all the segments of one leaf lamina (explant) and ultimately all of them developed into shoots. This number of differentiated shoots was quite high as compared to the report by Geetha *et al.* (1998), where number of differentiated shoots from the leaf was the lowest and to Eapen and George (1993) where differentiation of shoots was through leaf callus.

The leaf regenerated shoots had a tendency to callusing at the base whether they were subcultured individually or in groups and to necrosis of their apices and leaves. The shoot necrosis problem was circumvented in the presence of 758 μM $(NH_4)_2SO_4$ and 68 μM AdS, whereas the excessive callusing was contained with the drastic reduction in the concentration of BA from 2.22 μM to 0.04 μM. Furthermore, incorporation of CCC at the concentration of 63.25 μM still more improved the greenness of shoots. Thus, the nutrient medium BM2 in which the best growth of shoots and continued regeneration of shoots at each subculture was obtained comprised BM + 758 μM $(NH_4)_2SO_4$ + 0.04 μM BA + 0.57 μM IAA + 68 μM AdS + 63.25 μM CCC.

Rooting of isolated shoots was very easy and within a week 100 % rooting was obtained in the medium with 1.43 μM IAA and having a lower concentration of AdS (27 μM) than in BM2 (Fig. 1*F*). The rooted shoots processed through the acclimatization procedure survived more that 80 % after transplantation to pots with soil and grew normally in glasshouse (Fig. 1*G*). It took about 4 months to raise plantlets from the leaf explants, ready for transplantation to the soil and within a subsequent period of 1 month healthy plants were produced growing *ex vitro* in the soil.

In the present procedure, the use of young leaves, the segments of which were employed as explants ensures not only an ideal or most preferred organ for co-culture with the *A. tumefaciens* for genetic transformation studies, but also minimizes genetic variations at the explant level in contrast to regeneration protocol employing cotyledonary explants (Patel *et al.* 1994). At the same time, the *de novo* differentiation of multiple shoot meristems from the leaf lamina tissue offers enough opportunity for the incorporation of modified DNA in the meristem through the vector. Furthermore, since there is no intervening callusing during the process of shoot meristem differentiation, there are least chances of genetic variation creeping in the developing shoots other than those engineered through the vector, in contrast with several previous reports, where the regeneration of shoots has been obtained through callus phase (Kumar and Reddy 1983, Eapen and George 1993, George and Eapen 1994, Patel *et al.* 1994, Sreenivasu *et al.* 1998). That the callus is genetically instable is well-established and forms the basis of somaclonal variation (Skirvin 1978, Larkin and Scowcroft 1981). Further cloning of the transformed shoots in the same nutrient medium (BM2) afford to obtain a large number of shoots, which facilitate their evaluation for the modified traits in the shortest possible time. The number of cells or the sites available for modification by the vector per explant in the present system is far more than provided by a nodal explant (Prakash *et al.* 1994, Franklin *et al.* 1998) or cotyledonary node (Geetha *et al.* 1998).

The procedure reported here is far more practicable than those reported so far for a recalcitrant legume, like *C. cajan* and is also useful for increasing the number of facsimiles of genetically modified shoots in a short time, where the process of initiation of shoot meristems *in vitro* to the development of full-fledged plants in soil *ex vitro* is complete within a period of 5 - 6 months.

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