temperate regions where the moisture level is not so high.

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SUDIPTA MAJUMDAR
SOMENATH BANERJEE
K. K. DE*

Post Graduate Department of Botany, Darjeeling Government College, Darjeeling 734 101, India
**For correspondence.*

Efficient shoot regeneration in pigeon pea, *Cajanus cajan* (L) Millisp. using seedling petioles

Pigeon pea is a high-protein grain legume of semi-arid tropics and sub-tropics and its importance in Indian agriculture has been described time and again. This crop of strategic importance, is highly susceptible to attack by many insects and fungi. Attempts to obtain stress-resistant genotypes by conventional breeding methods have not been successful because of limited genetic variability amongst cultivated accessions and sexual incompatibility with wild relatives. Under such a situation, improvement of pigeon pea is possible only through genetic engineering and the essential prerequisite for achieving the goal is the development of an efficient *in vitro* regeneration system. Pigeon pea has been reported to be recalcitrant to regeneration in tissue culture. *In vitro* regeneration of pigeon pea has been reported earlier[1,2] and has recently been followed by many researchers[3]. Reports claiming high-efficiency shoot formation from explants have been obtained from experiments involved in multiplying existing meristem associated with cotyledonary region[4,5]. To our knowledge, no report describes high-efficiency regeneration of shoots *de novo* from cultured explants. It is also recognized that transformation by multiplication of existing meristem might result in chimeric transgenic plants that are difficult to handle. We report here a simple and efficient *de novo* regeneration from petioles explanted from young seedlings of pigeon pea.

Seeds of *Cajanus cajan* L. (ICP 26 and ICP 28) were obtained from the Genetic Resources Unit, International Crops Research Institute for Semi-Arid Tropics, Patancheru, Andhra Pradesh, India. Healthy and uniform seeds were aggregated and surface sterilized with 70% ethanol (v/v) for 1 min followed by treatment with 0.1% $HgCl_2$ (w/v) for 7 min. These seeds were rinsed with sterile distilled water 4–5 times and germinated aseptically on moist filter paper in magenta boxes. The leaves with petioles were excised from seven-day-old seedlings and used for *in vitro* regeneration studies. The basal medium[13] was supplemented with B5 vitamins[6], 2–5 mg l^{-1} of BAP, 0.1–0.5 mg l^{-1} NAA and 0.3 mg l^{-1} of IBA, either individually or in combination. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C for 15 min. Cultures were maintained at 26 ± 1°C under 16 h photoperiod. The entire leaves with petioles from seven-day-old seedlings were used for initiating cultures. A minimum of six explants were inoculated on the regeneration medium in 90 mm sterile petri plates. A minimum of 75 explants were used for each experiment and all experiments were repeated thrice. After every two weeks, the cultures were transferred to a fresh medium of the same composition. The number of responding cultures as well as the number of shoot buds/shoots per culture were recorded at regular intervals. Elongated and well-developed shoots were excised from shoot clump and transferred to rooting medium. Rooting of *in vitro* regenerated shoots was achieved on MS medium with 0.3 mg l^{-1} IBA[7,8]. Agargel was used instead of agar for rooting because of its purity and porosity.

Leaves whose petioles could be used as explants in the regeneration studies were obtained from seven-day-old seedlings, and hence petioles from such seedlings were used. Some earlier reports used leaf lamina as an explant for which leaves from 5–12-day-old seedlings were used[5,7,9]. However, the regeneration frequency in these studies was far from optimum. The explants, particularly the leaf lamina in the present study, expanded in size and white compact callus developed from the cut-end of the petiole. Within the first subculture, shoot-bud formation was observed all over the explant. The combination and concentration of growth regulators were crucial in getting a suitable regeneration response. The development of multiple shoots from explants started after four weeks of culture. Media were initially screened on the basis of the number of explants that responded in terms of shoot bud regeneration. Media that showed above 70% response in case of ICP 26 and 60% or above in case of ICP 28 were considered for further studies (Table 1), which included 2 mg l^{-1} BAP in combination with 0.1/0.2/0.5 mg l^{-1} NAA in case of ICP 28 and 4 mg l^{-1} BAP with 0.1 mg l^{-1} NAA in case of ICP 26. The media combinations, viz. 0.1 mg l^{-1} NAA with 2 mg l^{-1} BAP or 5 mg l^{-1} BAP, and 0.5 mg l^{-1} NAA with 4 mg l^{-1} BAP were considered in case of ICP 28. In case of ICP 26, the highest number of shoot buds per explant, i.e. 20 was observed in 0.5 mg l^{-1} NAA and 2 mg l^{-1} BAP. The elongation of shoot buds into proper shoots is another core factor in regeneration studies, and the highest number of average shoots, i.e. 11 was produced in 2 mg l^{-1} BAP in combination with 0.5 or 0.2 mg l^{-1} NAA (Table 2). The medium used for shoot-bud initiation was also continued for shoot devel-

opment in these experiments for both the cultivars, because new shoot-bud formation was observed on the explant randomly along with shoot elongation. ICP 28, on the other hand, had varied regeneration response, with the cultures initiated and grown in 0.1 mg l^{-1} NAA in combination with 5 mg l^{-1} BAP, and 0.5 mg l^{-1} NAA with 4 mg l^{-1} BAP giving the highest number of average shoot buds, i.e. 10. With the remaining media the response was lower. The average number of shoots per explant ranged from 2 to 6 (Table 3). The explants of ICP 26 inducing shoot buds and callus ranged from 0 to 91.6% on an average and in ICP 28, the explant response was a maximum of 71% (Table 1).

In general, the conversion of shoot buds produced from different explants into shoots was around 50% or even less in pigeon pea[8,10]. The ability to convert most of the shoot buds to shoots can be considered as an additional feature of the present protocol. We have observed in our study that 60% and above of the shoot buds developed into shoots (Tables 2 and 3). The response of the petiole decreased with an increase in the age of the leaf, i.e. the older the leaf, the lesser the response. This was in terms of the development of excessive callus with reduced number of shoot buds (data not shown). In cultivar ICP 26, the increase in BAP concentration by keeping NAA concentration constant did not result in an increase in the number of shoots that got regenerated but resulted in additional callus. In this cultivar, the average number of shoots had increased with an increase in NAA concentration from 0.1 to 0.5 mg l^{-1}, by keeping the BAP levels at 2 mg l^{-1}. In the genotype ICP 28 the variation is random, which could be due to the physiological status of the explants and their endogenous hormone levels. The explants from this cultivar were basically slow-growing in culture, with the maximum culture response of explants on any combination of growth regulators being 71%; the average number of shoots ranged from 2 to 6 only (Table 3). The maximum response was achieved on 0.1 mg l^{-1} NAA with 5 mg l^{-1} BAP. The frequency of rooting was high in 0.3 mg l^{-1} IBA, followed by different concentrations of NAA[5] and IAA[7,8]. The elongated shoots were rooted in MS medium supplemented with 0.3 mg l^{-1} IBA. Plantlets could be hardened in small pots with a mixture of red soil, vermiculate and

Table 1. Response of petiole explants in percentage of ICP 26 and ICP 28 on MS medium with different combinations of plant growth regulators

	ICP 26		ICP 28	
Supplement (mg l^{-1})	Shoot buds + callus	Average no. of shoot buds per explant	Shoot buds + callus	Average no. of shoot buds per explant
0.1NAA + 2BAP	73.0 ± 2.0	12.0 ± 0.1	65.0 ± 5.0	6.1 ± 0.1
0.1NAA + 3BAP	58.3 ± 8.6	12.1 ± 0.5	26.6 ± 8.9	8.0 ± 1.0
0.1NAA + 4BAP	91.6 ± 8.4	13.3 ± 0.3	15.0 ± 5.0	14.8 ± 0.3
0.1NAA + 5BAP	29.2 ± 5.5	8.8 ± 0.25	71.8 ± 5.5	10.3 ± 0.7
0.2NAA + 2BAP	75.9 ± 6.5	18.0 ± 0.2	33.3 ± 8.9	15.3 ± 0.3
0.2NAA + 3BAP	58.0 ± 8.5	17.8 ± 0.2	52.7 ± 2.7	7.3 ± 0.5
0.2NAA + 4BAP	44.4 ± 8.1	5.7 ± 0.35	26.8 ± 1.8	7.0 ± 0.5
0.2NAA + 5BAP	46.5 ± 3.5	22.8 ± 0.5	29.0 ± 4.0	11.3 ± 0.3
0.5NAA + 2BAP	70.8 ± 5.8	20.8 ± 0.3	37.5 ± 0.3	5.2 ± 0.2
0.5NAA + 3BAP	52.7 ± 2.7	16.6 ± 1.1	47.0 ± 3.0	5.5 ± 0.3
0.5NAA + 4BAP	44.4 ± 8.1	32.1 ± 0.5	63.0 ± 3.0	10.2 ± 0.2
0.5NAA + 5BAP	0.0 ± 0.0	0.0 ± 0.0	42.2 ± 2.2	10.8 ± 0.3

Table 2. Conversion of shoot buds of ICP 26 into shoots on MS medium with different combinations of plant growth regulators in percentage

Supplement (mg l^{-1})	Average no. of shoot buds	Average no. of shoots	Percentage of conversion
0.1NAA + 2BAP	12.00	8.7 ± 0.6	72
0.1NAA + 4BAP	13.30	6.8 ± 1.2	51
0.2NAA + 2BAP	18.00	11.2 ± 2.0	62
0.5NAA + 2BAP	20.80	11.2 ± 1.4	53

Table 3. Conversion of shoot buds of ICP 28 into shoots on MS medium with different combinations of plant growth regulators in percentage

Supplement (mg l^{-1})	Average no. of shoot buds	Average no. of shoots	Percentage of conversion
0.1NAA + 2BAP	6.15	2.7 ± 0.5	43
0.1NAA + 5BAP	10.30	6.6 ± 1.2	64
0.2NAA + 3BAP	7.30	5.0 ± 0.7	68
0.5NAA + 3BAP	5.45	4.0 ± 0.5	73
0.5NAA + 4BAP	10.00	5.0 ± 1.0	50

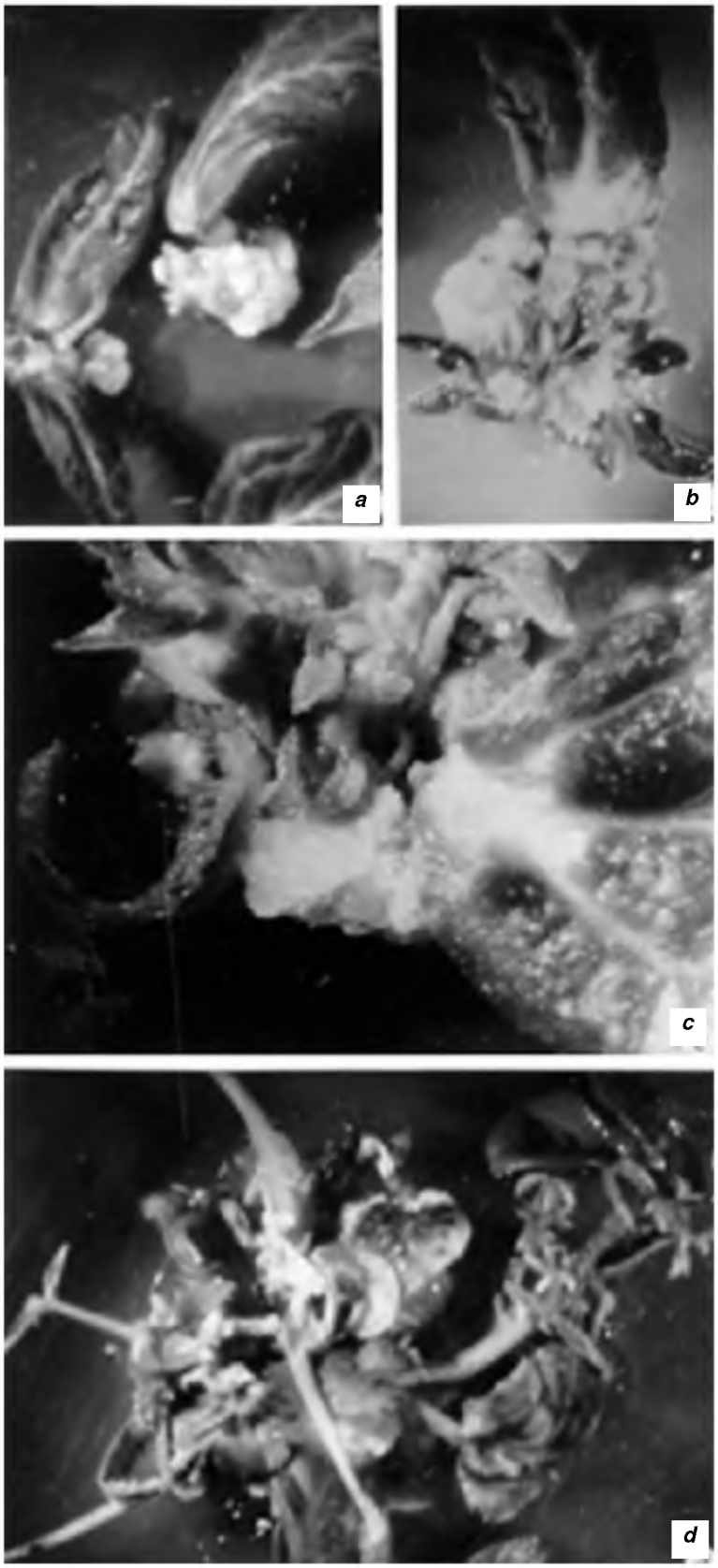

Figure 1. Different stages of *in vitro* regeneration of pigeon pea from petiole. ***a***, Callus and shoot bud formation from petiole. ***b***, Callus and shoots developing from petiole. ***c*** and ***d***, Growing shoots of ICP 26 on MS medium containing 2.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA.

farmyard manure in a ratio of 1 : 1 : 1 and transferred to the soil.

This simple protocol is rapid and efficient compared to earlier protocols. The maximum average number of shoots in the previous findings ranged from 3.75 to 8.0 (refs 7, 11, 12), apart from that of Shiva Prakash *et al.*[4], who claimed 43 ± 8.9 shoots. The latter report showing higher shoot-formation frequency was based on the multiplication of existing meristem from the cotyledon region and not because of *de novo* formation. Our protocol is based on *de novo* regeneration of shoot buds from the distal cut-ends of the petioles with high efficiency and is highly suitable for genetic transformation.

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T. SRINIVASAN
VINOD K. VERMA
P. B. KIRTI*

Department of Plant Sciences,
University of Hyderabad,
Hyderabad 500 046, India
**For correspondence*
e-mail: pbksl@uohyd.ernet.in