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Multiple shoot induction by benzyladenine and complete plant regeneration from seed explants of chickpea (*Cicer arietinum* L.)

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Abstract The efficacy of benzyladenine (BA) to induce multiple shoots from seed explants of chickpea (*Cicer arietinum* L.) was assessed. Shoot differentiation was influenced by the type of seed explant, genotype and concentration of BA. Orientation of the explant also strongly influenced the shoot regeneration response. The optimum BA concentration for shoot/shoot bud regeneration was genotype dependent. Two types of BA-induced response were observed: (1) at less than 7.5 μM BA, direct shoot differentiation (2 to 4-cm-long shoots) was observed within 30 days; (2) at higher BA concentrations (75–100 μM), shoot/shoot bud differentiation was achieved in 45–90 days. A high BA concentration inhibited subsequent rooting of shoots. Roots, however, could be easily induced on shoots derived from <12.5 μM BA. Following transfer to soil, 80% of the regenerants developed into morphologically normal and fertile plants.

Key words *Cicer arietinum* · Shoot regeneration · Benzyladenine

Abbreviations *BA* Benzyladenine

Introduction

Successful application of tissue culture techniques often requires efficient production of multiple shoots. Among the grain legumes, in vitro production of multiple shoots from seed/cotyledon explants has been achieved in mungbean (Gulati and Jaiwal 1990, 1994), peanut (McKently et al. 1989), *Phaseolus vulgaris* (Malik and Saxena 1992a), pea, chickpea, lentil (Malik and Saxena 1992b) and pigeonpea (Prakash et al. 1994).

In chickpea, thidiazuron was shown to induce multiple shoots (Malik and Saxena 1992b). Benzyladenine (BA) at a high concentration was also shown to be effective in shoot regeneration in *P. vulgaris* (Malik and Saxena 1992b). Recently, the efficacy of BA in inducing multiple shoots was also demonstrated in mungbean (Gulati and Jaiwal 1994) and pigeonpea (Prakash et al. 1994). In the present study, BA, a relatively inexpensive cytokinin, was tested for its efficacy to induce multiple shoots in chickpea.

Materials and methods

Seed material

Seeds of chickpea varieties BG-362, BG-329, BG-267, BG-256 and C-235 were obtained from the Division of Genetics, Indian Agricultural Research Institute, New Delhi, India. Healthy chickpea seeds of uniform dimension were agitated thoroughly in a dilute solution of Tween 20 for 2 min. Seeds were then rinsed under running tap water. Seeds were then surface sterilized with 0.1% mercuric chloride for 3 min, followed by repeated washings (5–6 times) with sterilized double-distilled water. They were germinated aseptically on filter paper moistened with sterile distilled water.

Seed explants

The following explants were prepared after 2 and 3 days: (A) embryo with intact embryonic axis and both cotyledons; (B) same as (A) except the emerging radicle tip (5 mm) was excised; (C) embryo with only one cotyledon along with an intact embryonic axis; (D) same as (C) except the emerging radicle tip (5 mm) was excised. It should be noted that in 2–3 days, the emerging embryonic axis was the radicle. It was presumed that preventing radicle growth might stimulate multiple shoot production.

Culture medium

All embryo/shoot tip explants were cultured on medium containing MS salts (Murashige and Skoog 1962), vitamins of Gamborg (Gamborg et al. 1968), 3% sucrose and 0.8% agar. This medium, referred to as modified MS medium, was supplemented with various concen-

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trations of BA. The pH of the medium was adjusted to 5.8 prior to autoclaving. Fifteen replicates for each explant/treatment were evaluated.

BA treatments

Initially the effect of various concentrations of BA (2.5, 5.0, 7.5, 10, 12.5, 25, 37.5 and 50 μM) were tested on two cultivars, BG-362 and BG-329. The effect of explant orientation on multiple shoot production was investigated with cv. BG-256. The 2.5 μM BA concentration was chosen as it was found to be generally best from previous experiments. At higher concentrations of BA, >12.5 μM, browning of cultures, less vigorous thin shoots and shoot tip decay were observed. For three more genotypes included later in this study, the BA concentration response was studied up to 12.5 μM. As the present protocol did not show any response beyond 12.5 μM BA, the protocol described for *P. vulgaris* by Malik and Saxena (1992a) was employed to test the higher concentration effect of BA. The basic difference between these two protocols was that the chickpea explants were subjected to dark incubation for 5 weeks. In the second set of experiments, BA concentrations were tested in the range 75–150 μM. Seeds were immersed in undiluted concentrated sulfuric acid for 60 s to soften the waxy seed coat, and washed twice with sterile water. These seeds were then immersed in ethanol for 1.5 min and then in a 1% solution of sodium hypochlorite for 20 min. During this period they were continuously stirred with a magnetic bar. These additional sterilization steps were included as part of the protocol of Malik and Saxena (1992a). Later studies indicated that they were not necessary. Further seed sterilization/inoculation procedures were as described above. In this set of experiments, five seeds were cultured in 250-ml conical flasks containing 50 ml of the culture medium supplemented with various concentrations of BA.

Histology

Seed explants cultured on 5.0 μM BA as described above were fixed in formaldehyde, glacial acetic acid and (70%) ethanol (2:1:17 vol/vol). The explants were serially dehydrated with ethanol and tert-butanol, and then infiltrated and embedded in paraffin wax. Serial longitudinal sections of 8 μm thickness were cut using a rotary microtome. These sections (ribbons) were mounted on glass slides and passed through a series of deparaffinizing solutions. The sections were examined under a light microscope with or without staining with haematoxylin.

Culture conditions

Cultures were maintained in a 16 h light/8 h dark photoperiod, at 25±1°C. Phillips (India) day and night fluorescent tubes were used to obtain 60 μmol $m^{-2}s^{-1}$ light intensity. In the experiment in which seeds were inoculated in 250-ml conical flasks, cultures were initially incubated at 25°C in darkness for the first 5 weeks and then transferred to the photoperiod described above. Except for this deviation, all other culture conditions were the same as those given by Malik and Saxena (1992a). Cultures were maintained up to 90 days.

Rooting and establishment

Shoots (2–3 cm) were excised from various types of explants derived from <12.5 μM BA. Following the protocol of Polisetty et al. (1996), excised shoots were initially cultured with 1/4-strength MS medium + 0.75% sucrose + 0.8% agar but without further hormonal supplement for 30 days. With this procedure, rooting was obtained in 90–100% shoots. Later, these plantlets were transferred to 1/4-strength MS medium described above but without agar. Plantlets were supported by moistened paper immersed in culture tubes containing 20 ml medium. This culture facilitated increased root growth. After 10 days culture, the plantlets were processed for 1/4-strength Hoagland culture for 20 days and later establishment in soil/vermiculite following the protocol of Polisetty et al. (1996).

Statistical analysis

Twenty replicates for each seed explant/treatment were evaluated. Replicated data of all experiments were statistically analyzed using a completely randomized block design, and means were evaluated at the P=0.05 level of significance using Duncan's multiple-range test.

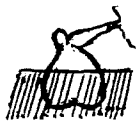
Results and discussion

Using 5 μM BA, the effect of the type of explant on shoot regeneration was examined in cv. BG-362 (Table 1). Preliminary experiments with this cultivar had shown that shoot number was highest when the seed explant was 2–3 days old (data not shown). Hence, the four different types of seed explant were tested at 2 and 3 days. The maximum number of multiple shoots was observed using 2-day-old explant type B (i.e., seed with both cotyledons in which the radicle tip (5 mm) was excised (Table 1). Excision of the radicle tip contributed to a significant increase in the number of shoots only in 2-day-old explants. No such effect was observed in 3-day-old explants. Similar observations of plant regeneration using cotyledon explants with intact cotyledonary nodes reported among the legumes included *Cajanus cajan* (Mehta and Mohan Ram 1980; Prakash et al. 1994), *P. vulgaris* (Malik and Saxena 1992a), *Vigna mungo* (Gill et al. 1987), *V. radiata* (Mathews 1987; Gulati and Jaiwal 1994) and *Cicer arietinum* (Brandt and Hess 1994). However, the basal medium, and growth regulator source and concentration were different in each case. McClean and Grafton (1989) used bean cotyledonary node explants in which axillary buds were removed by scraping the axis and its surrounding region prior to culture on a medium supplemented with BA (5 μM). It was proposed that axillary bud excision was responsible for adventitious

Table 1 Effect of explant type and age on multiple shoot regeneration in chickpea using modified MS medium supplemented with 5.0 μM BA. Cv. BG-362 cultured for 30 days after inoculation. Means ±SE followed by the same letter are not significantly different at the 5% level by Duncan's new multiple-range test

Explant type	Age or explant (days)	Number of shoots/explant	
		Control (– BA)	Treatment (+ BA)
(A) Embryo explant with intact embryonic axis and both cotyledons	2	2.0	13.3±3.8[b]
	3	1.9	11.9±1.5[b]
(B) As in A except the tip of the radicle (5 mm) was excised	2	2.1	20.0±1.0[a]
	3	1.5	9.5±1.6[b]
(C) explant as in A except one of the cotyledons was excised	2	1.6	9.4±1.6[b]
	3	1.7	11.0±2.6[b]
(D) explant as in C except tip of the radicle was excised	2	1.8	14.3±2.8[b]
	3	1.2	9.6±3.0[b]

Table 2 Effect of explant orientation on shoot regeneration in chickpea cv. BG-256 using modified MS medium supplemented with 2.5 μM BA, type B explant (see Table 1), cultured for 25 days. Means ±SE followed by the same letter are not significantly different at the 5% level by Duncan's new multiple-range test

Orientation of seed explant	Schematic description	Number of shoots/explant
(1) Pointed end of the embryo outside medium; rest of the embryo dipped in medium		6.0±0.4[a]
(2) Pointed end dipped into medium; rest of the embryo outside the medium		1.0±0.2[c]
(3) Mycropylar side facing the medium		6.4±0.5[a]
(4) Mycropylar side facing away from the medium		4.8±0.3[b]

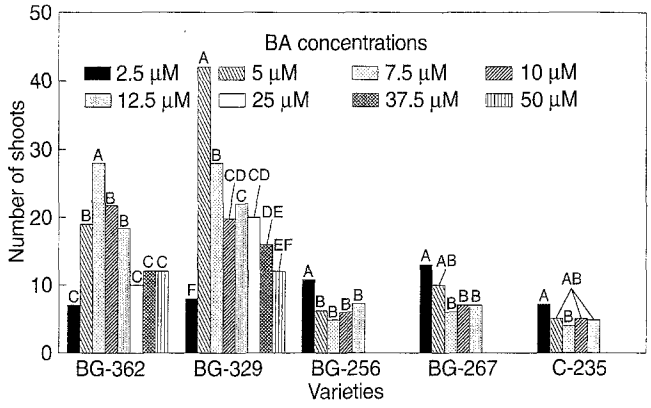


Fig. 1 The effects of genotype and BA concentration on multiple shoot regeneration in seed explants of chickpea. Each bar represents the mean of 15 replications. Within each cultivar, bars indicated by similar letters are not significantly different at $P=0.05$ (Duncan's new multiple-range test). Culture medium: modified MS medium supplemented with BA. Growth period: 25 days

meristem initiation. However, in our experiment, only the radicle tip was excised.

Excision of one of the cotyledons while keeping the embryonic axis intact does not appear to adversely affect the number of shoots produced per explant (Table 1). Gulati and Jaiwal (1990, 1994) demonstrated that in *V. radiata*, cotyledon size affected the regeneration ability of the explant. Their later study clearly demonstrated the requirement of the embryonic axis along with cotyledons for inducing multiple shoots. Examining the effect of BA concentrations on the shoot production potential of five different cotyledon explants of peanut, McKently et al. (1989) concluded that cotyledons having intact embryos produced more shoots than other explant types, particularly those in which embryo axes were excised.

Evidently the presence of cotyledons was essential for realising maximum shoot production potential. If the shoots are primarily arising from the existing buds of the embryonic axis, the shoot number should not decrease when both the cotyledons are removed. Our preliminary experiments showed that multiple shoots were not produced when only the embryonic axis with axillary bud, but without cotyledons, was used as explant. In such explants, only 1–2 shoots were observed. However, when similar explants were used in another study, not a single shoot developed (Franklin et al. 1991). Hence, we used explant type B which contained both cotyledons along with an intact embryonic axis.

Wide variation in the number of shoots per explant occasionally encountered within a treatment prompted us to study the influence of seed explant orientation on shoot production. This was investigated with cv. BG-256 using 2.5 μM BA and explant type B. The maximum shoot number was observed when the explant was inoculated with the micropylar surface facing the medium (Table 2). However, if the micropylar end was dipped in the medium (treatment 2) only a single shoot was produced (Table 2). Thus, part of the variability previously encountered within a treatment could be attributed to explant orientation.

The effect of various concentrations of BA on shoot production was initially tested in two cultivars. The maximum number of shoots was observed in BG-329 and BG-362 using 5.0 and 7.5 μM BA, respectively (Fig. 1). However, at higher BA concentration (25, 37.5 and 50 μM), characteristic symptoms observed were browning, thin weak shoots and shoot tip decay. Shoot production was not significantly improved using BA at concentrations above 12.5 μM (Fig. 1). Therefore, three other cultivars included in a later study were tested only up to 12.5 μM BA. For these three cultivars, the optimum concentration was 2.5 μM (Fig. 1).

In experiments in which lower (<12.5 μM) BA concentrations were used, a few shoots arising from the explants were observed to develop inside the medium. Such shoots exhibited enhanced axillary branching. To exploit this phenomenon, 4-day-old explant type B of cultivar C-235 was used. When the explant was inoculated on BA medium, the position was reversed, i.e., the growing shoot tip was kept inside the medium. This resulted in extensive axillary branching (Fig. 2). Closer observation revealed a cluster of shoots arising at the base of each node.

In chickpea, Barna and Wakhlu (1993) did not observe any shoot regeneration using cotyledons as explants. Brandt and Hess (1994) regenerated about seven shoots from each cotyledonary node explant cultured at 4.4 μM BA; however, no quantitative data were presented. These studies may explain, in part, the wide variation in regeneration potential particularly at low BA concentrations re-

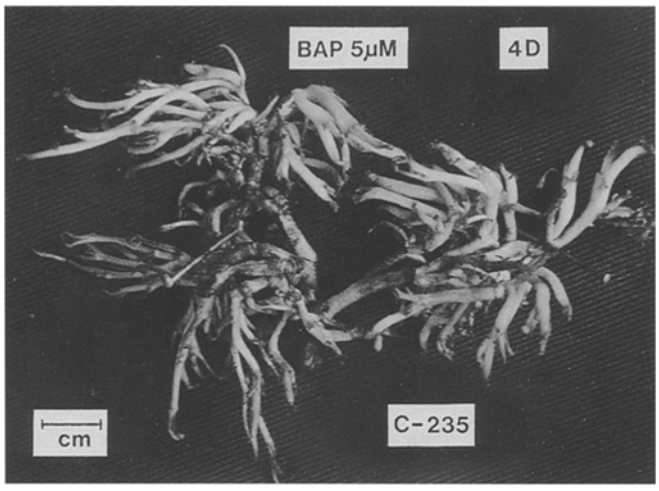


Fig. 2 Multiple shoots regenerated from 4-day-old type B explant (see Table 1) cv. C-235 inoculated keeping the emerged shoot tip inside the modified MS medium supplemented with 5.0 μM BA. Photograph taken at 25 days after inoculation

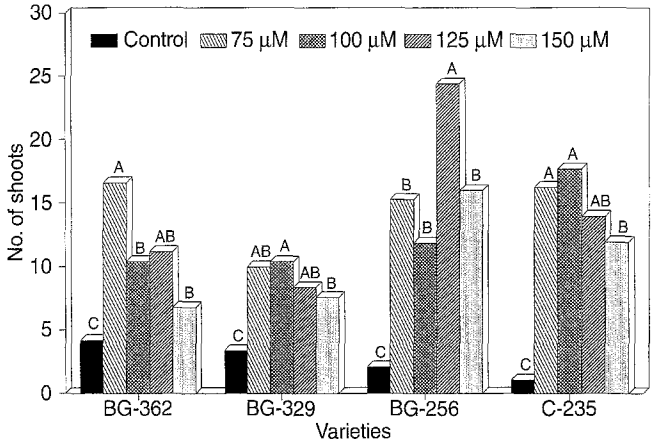


Fig. 4 The effects of genotype and higher BA concentrations on shoot bud regeneration in seed explants of chickpea. Each bar represents the mean of 20 replications. Within each cultivar, bars indicated by similar letters are not significantly different at P=0.05 (Duncan's new multiple-range test). Culture medium: modified MS medium supplemented with BA. Growth period: 90 days

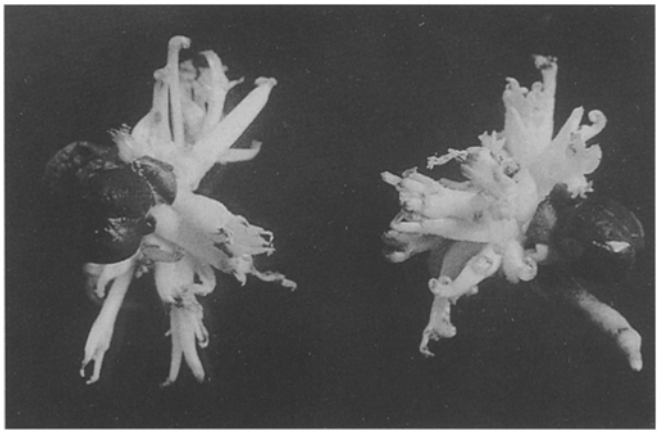


Fig. 3 Multiple shoot regenerated from seed explants of cv. BG-362 cultured on modified MS medium supplemented with 100 μM BA. Initially the explants were cultured in the dark for 5 weeks and later transferred to the light. Photograph taken at 45 days after inoculation

ported among genotypes (Rubluo and Kartha 1985; McKently et al. 1989; Gulati and Jaiwal 1990, 1994). The data indicate that in chickpea, the shoot regeneration response is not only dependent upon genotype but also on BA concentration. Thus, it is desirable to determine the optimum concentration of BA when a specific genotype is targeted for regeneration, for example, to develop transgenic plants.

In *P. vulgaris*, the maximum shoot regeneration response was reported using 10 μM thidiazuron and 80 μM BA (Malik and Saxena 1992a). However, in chickpea in the present study, no BA concentration beyond 12.5 μM was found beneficial in enhancing the shoot regeneration potential. So the protocol of Malik and Saxena (1992a) was adopted to test the efficacy of four BA concentrations among five genotypes of chickpea for shoot bud/shoot induction. The basic difference between our protocol and the previous protocol was the initial culture of explants in darkness. Our preliminary experiments indicated that main shoot and root growth of these seed explants were severely inhibited at high BA levels and under dark incubation conditions. It was presumed that severe inhibition of shoot growth under dark culture would stimulate multiple shoot regeneration. Seeds of cv. BG-267 did not germinate. However, the control (i.e., no BA) gave normal germination. No known reason, except the high BA concentration, could be attributed for the less than 5% germination observed in BG-267. Even in the few seeds which did germinate, no shoot bud differentiation was observed. Hence, the data of BG-267 were omitted. At BA concentrations >75 μM, the multiple shoot regeneration response was slightly different. Growth of the main shoot and axillary buds were stunted. In about 45 days after inoculation, a cluster of shoot buds appeared from the base of the main shoot and axillary buds (Fig. 3).

Because of the very high concentration (100 μM) of BA, green shoot buds which developed on these shoots by 90 days did not grow beyond 4–5 mm. This is in contrast to the 3–4 cm achieved at lower BA concentrations. Our data support the suggestion by Cheng et al. (1980) that higher concentrations of BA would stimulate bud formation but inhibit bud growth. A higher BA concentration did enhance the production of numerous shoot buds (Fig. 3) in all explants. At 90 days after inoculation, only shoot buds which were larger than 4 mm were counted. In cv. BG-329 and BG-362, shoot numbers were much higher at low BAP levels. However, a nearly 100% increase in shoot buds was observed for BG-256 and C-235 in comparison to lower concentrations (compare Fig. 1 and Fig. 4). The optimum concentration was also observed to be genotype dependent (Fig. 4).

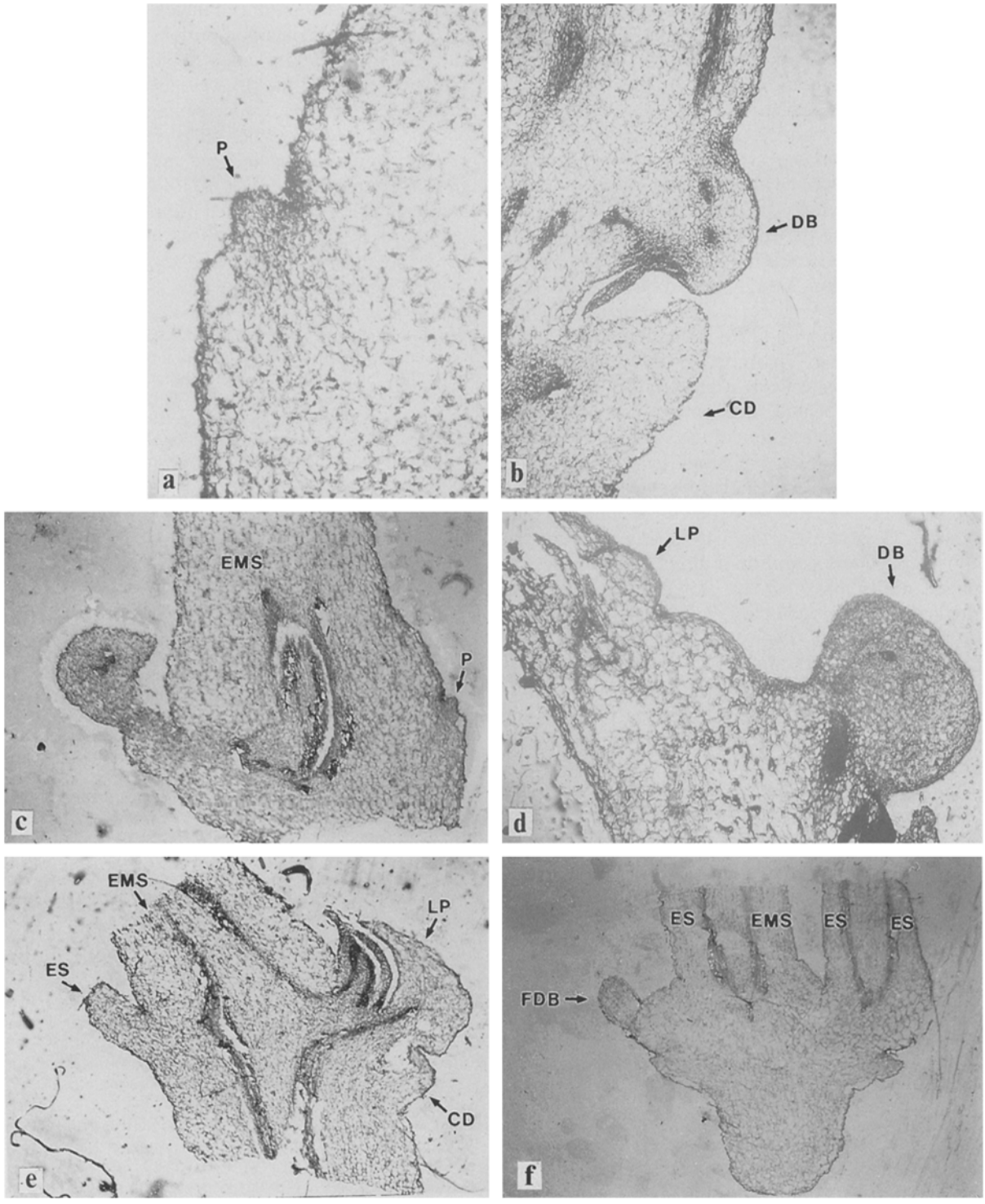


Fig. 5a–f Microphotographs of longitudinal sections of type B explant of cv. BG-362, *Cicer arietinum* L. showing multiple shoot formation. Culture medium: modified MS medium supplemented with 5 μM BA (*P* protuberance, *DB* developing bulge, *FDB* fully developed bulge, *LP* leaf primordia, *EMS* excised main shoot, *ES* excised shoot, *CD* cotyledonary node axis. **a** Protuberance appearing on the basal portion of the shoot near the cotyledonary node axis. **b** Developing bulge. **c** Bulge transformed to shoot bud. **d** Differentiated shoot. **e** Complete longitudinal section of 6-day-old explant showing developed shoot and differentiating shoot. **f** Complete longitudinal section of 13-day-old explant. Four fully emerged shoots (excised) and initiation of two new shoot buds. Note the bulge on the right side and bulge transformed into a bud (left side)

Efforts are being made to enhance the number of shoots developed from induced shoot buds. If more than 50–100 shoots per seed explant can be achieved, this system may be ideal for employing microprojectile technology to obtain transgenic plants. Even at the present rate of shoot production per explant, our protocols are potentially amenable for microprojectile or *Agrobacterium*-mediated techniques for the production of transgenic plants.

In our experiments, shoot buds developed either at the base or junction of the regenerated shoots. The physical

position and shoot emergence pattern suggested an axillary origin. To understand the origin and type of these shoots, anatomical studies were carried out using variety BG-362 and explant type B, cultured at 5 μM BA. Longitudinal sections were taken at 3–13 days after excising the regenerated shoots. Light microphotographs (Fig. 5a–f) indicated that adventitious shoots arose sequentially from the basal peripheral region of an already emerged shoot which was adjacent to a cotyledonary node. Initially, small protuberances emerged on the epidermal cell layer at the basal portion of the already developed shoot (Fig. 5a), which later developed into a bulge (Fig. 5b) and subsequently differentiated into a shoot (Fig. 5c, d). This evidence coupled with physical observation indicated that no more than two shoots would arise at a given time. Once the shoot attained 1–1.5 cm in length, the cycle of initiation and development of new shoots was repeated. This pattern was confirmed from sections taken as a function of days after inoculation. At 6 days after inoculation, one shoot in addition to the main shoot had emerged and another was in the process of development (Fig. 5e). New shoot bud development could be seen at 10–13 days after inoculation (Fig. 5f). The induction of new shoots could be a direct consequence of BA-induced inhibition of shoot growth. The extensive branching pattern observed in Fig. 2 also supports the suggestion that BA inhibits shoot growth and also promotes adventitious/axillary bud initiation. These microphotographs confirm that new adventitious buds arose from peripheral epidermal cells but not from the pre-existing buds. These shoot buds have no visible connection with the original vascular tissue. However, no anatomical studies were done to understand the extensive axillary-type branching pattern observed in Fig. 2. The appearance of more than two shoots at each node suggested an adventitious origin. However, an axillary type of origin was not ruled out.

The possibility of apical dominance inhibiting the development of adventitious buds on cotyledonary node explants in soybean was suggested by Cheng et al. (1980). In the present experiments, in the control treatment (i.e., minus BA), the main shoot emerged in 4 days and developed into a seedling in 7 days, suppressing the emergence of adventitious buds. In BA-treated seed explants (especially when the BA concentration was below 12.5 μM), the main shoot had emerged in 4 days, but its growth was suppressed. Even at 25 days after its emergence, its length was less than 4 cm. This suppression of main shoot growth could have resulted in adventitious bud development. Experimental data presented by Gambley and Dodd (1990) also support this hypothesis. They demonstrated that if the apical dominance of an existing axillary bud is eliminated physically, a large number of shoots could arise in response to cytokinin treatment. Histological evidence of such an adventitious/axillary origin of multiple shoots was reported in other crops in which a cotyledonary node with axillary bud was used as explant, e.g., *V. radiata* (Gulati and Jaiwal 1994), *C. cajan* (Prakash et al. 1994), *P. vulgaris* (Franklin et al. 1991; Malik and Saxena 1992a).

Multiple shoots produced under various treatments did not produce any roots upon prolonged culture on BA medium. For rooting, only shoots obtained from seed explants cultured with <12.5 μM BA were used. In our earlier study, we demonstrated that the percentage of cultures forming roots on excised shoots of chickpea could be vastly improved by reducing the nitrogen and sucrose concentration in MS medium (Polisetty et al. 1996). Further rooting and establishment were achieved following the protocol of Polisetty et al. (1996). Following transfer to soil, 80% of the regenerants developed into plants, which were morphologically normal and fertile.

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