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The role of cotyledonary tissue in the differentiation of shoots and roots from cotyledon explants of *Brassica juncea* (L.) Czern.

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Abstract

In cotyledon cultures of *Brassica juncea*, shoots and roots invariable differentiate at the cut end of the petiole. Organogenesis occurred only if the proximal cut end of the petiole was in contact with the medium. In the absence of the petiole, differentiation from the lamina was rare. Hence investigations were carried out to study the influence of the cotyledonary lamina on regeneration of shoots and roots from the petiolar cut end. The lamina tissue was surgically removed from the cotyledon explants at different durations (0–10 days) after culturing them on either root-forming (basal medium) or shoot-forming (basal medium containing 5.0 μM N^6-benzyladenine) media. The differentiation of roots or shoots from the petioles was dependent on the presence of the lamina for at least 7 days of culture. Quantitative removal of the laminar tissue had a corresponding negative effect on shoot bud differentiation from the petiole. The nature of the 'lamina factor' was found to be auxin-like.

Introduction

Regeneration of plants directly from the explant has been used for in vitro propagation of herbaceous and woody species [4]. Such regenerants have also proved to be a valuable source of somaclonal variants [3, 19]. Plant regeneration from explants can also serve as an efficient system to obtain transgenic plants, where isolated cells do not express cellular totipotency [6, 11].

Several reports of plant regeneration from seedling explants of *Brassica* species have been published during the past decade. However, there is considerable variation in the observations of different groups, even when dealing with the same species. For example, Lazzeri & Dunwell [9] and Murata & Orton [13] observed that cotyledon explants of *Brassica oleracea* exhibit poor and sporadic differentiation, but Horeau et al. [5] have reported high frequency regeneration from excised cotyledons of three varieties of this species. Moreover, a great deal of variability in shoot formation from cotyledons of several *Brassica* species is attributable to experimental replications, as was apparent from studies of Murata & Orton [13]. While considerable attention has been paid to the manipulation of culture medium and selection of genotypes to improve the in vitro plant regeneration response, the nature of the explant has been largely ignored. The results reported in this paper highlight how critical this factor is for achieving consistently high frequency regeneration in cotyledon cultures of *Brassica juncea*.

Materials and methods

The seeds of *Brassica juncea* (L.) Czern. cv. RIK-81-1 were obtained through the courtesy of Mr.

Explant	Portion severed
E1 (control)	Entire cotyledon was cultured
E2	Complete petiole
E3	The petiole along with a small portion of the lamina
E4	The petiole and the lower half of the lamina
E5	One lobe of the lamina
E6	Both the lobes of the lamina
E7	Upper half of the lamina
E8	The entire lamina
E9	Longitudinal half of the cotyledon including petiole

S.D. Dubey, Regional Research Station, Indian Agricultural Research Institute, Kanpur. Aseptically germinated 5-day-old seedlings served as the source of cotyledon explants. To raise the seedlings, seeds were given a quick rinse in 90% ethanol and then surface sterilized with 0.2% (w/v) mercuric chloride for 7 min. After rinsing 3 times in sterile distilled water, 5–8 seeds were aseptically implanted in each culture tube (150 mm × 25 mm) containing basal MS medium [12] consisting of MS salts, vitamins, 3% sucrose and 0.8% Difco Bacto agar. The tubes were plugged with non-absorbant cotton wrapped in one layer of cheesecloth and maintained at 25 ± 2° C under continuous light of 80 μmol $m^{-2} s^{-1}$ irradiance provided by fluorescent lamps.

Basal MS medium was used for root formation and this MS medium supplemented with 5.0 μM N^6-benzyladenine (shoot induction medium; SIM) was used for shoot formation. Unless specified otherwise, a cotyledon explant consisted of a bilobed lamina and ca. 2 mm long petiole, and it was planted with its abaxial surface in contact with the medium and the proximal cut end of the petiole embedded into the medium. Details of selection of these conditions are described elsewhere [17].

To study quantitative relationships between the amount of cotyledonary tissue removed and the frequencies of shoot bud differentiation, the following explants were prepared by severing different portions of the cotyledon and cultured on SIM with the petiolar end of the explant embedded in the medium.

In a separate study, the cotyledonary lamina was removed from root-forming as well as shoot-forming cultures at different stages in culture to study the influence of the lamina on the organ differentiation process. Attempts were also made to substitute for the lamina with different concentrations of IAA (filter-sterilized) in the shoot-forming medium.

All cultures were maintained at 25 ± 2 °C under continuous light of 80 μmol $m^{-2} s^{-1}$ irradiance provided by fluorescent lamps (TL 40 W/54 cool daylight). Usually 48 cultures were used per treatment and each experiment was repeated at least twice. The cultures were examined periodically and the morphological changes were recorded on the basis of visual observations. The effects of different treatments were quantified on the basis of percent cultures showing the response. The data pertaining to frequencies of root or shoot bud differentiation were subjected to the chi square test for homogeneity of proportions and significant treatment differences selected by the Post Hoc Multiple comparisons test [10]. In each treatment, numbers followed by same letters are not significantly different at the $p = 0.05$ level.

Observations

In cultures of excised cotyledons, the differentiation of roots and shoots always occurs at the proximal cut end of the petiole that is in contact with the medium [17]. This observation raises two questions: (1) does the laminar tissue lack the potential to form shoots, and (2) is the lamina essential for shoot differentiation from the petiolar tissue? To answer these questions, various explants were prepared by severing different portions of the cotyledons prior to culture on SIM (see Materials and methods and Fig. 1).

The responses of these various explants are summarized in Fig. 1. The lamina alone (E2) exhibited shoot formation (Fig. 2C), but the frequency of differentiation was significantly lower than that in the control (E1; Fig. 2A). More interesting to note is that the petiole devoid of lamina (E8) showed a much poorer response (Fig. 2B) than the lamina alone (E2, Fig. 2C). The frequency of differentiation from the petiole was enhanced with an increase in the amount of the lamina left intact with it (E8 < E7 = E6 < E5 < E1). These observations suggest that the cells most competent to differentiate shoots are located at the base of the petiole (Fig. 2A, D), but to express their totipotency they are dependent on some factor(s) from the lamina. The unknown morphogenic factor seems to be largely provided by the two lobes of the

Explant	Treatment*	% Response	No shoots/explant
E_1		70.8^d	6.1 ± 1.7
E_2		31.8^c	4.3 ± 1.7
E_3		12.5^b	1.5 ± 0.7
E_4		4.2^a	1.2 ± 0.3
E_5		58.3^d	5.0 ± 1.5
E_6		37.5^c	4.2 ± 1.6
E_7		37.5^c	1.2 ± 0.3
E_8		4.2^a	1.8 ± 0.9
E_9		62.5^d	1.3 ± 1.0

* Shaded portion was severed

Fig. 1. Quantitative relationship between removal of cotyledonary tissue (shaded portion in column 2) and shoot bud differentiation (columns 3 & 4) on MS + 5.0 μM BA in *Brassica juncea*. Growth period: 3 weeks. Frequencies followed by same letters are not significantly different at $p = 0.05$ (Post Hoc Multiple Comparisons test). $\pm$ represents standard error of mean.

lamina because the reduction of shoot bud differentiation caused when both the lobes (E6) were removed is equal to the reduction caused by severing of the upper half of the lamina (E7). The absence of only one lobe (E5) did not cause significant reduction of bud formation. Removing the two lobes not only reduced the percentage of cultures forming shoots, but also brought about a proportional reduction in the number of shoots per responding explant. These findings suggest a quantitative relationship between the amount of lamina present and the degree of budding. Another noteworthy observation was that although the excised lamina (E2) showed a higher regeneration potential than the excised petiole (E8), the morphogenic potentiality of the lamina was not expressed in the presence of the petiole (E1, E9; Fig. 1). This may be due to a strong gradient of some endogenous factor(s).

Having established that the lamina plays a crucial role in the differentiation of shoot buds from the petiolar cells, an experiment was done to determine the critical period during which the presence of lamina was essential for the petiolar cells to express their totipotency. For this the entire cotyledon explant (E1) was cultured on the basal MS medium and the SIM, and the lamina was removed

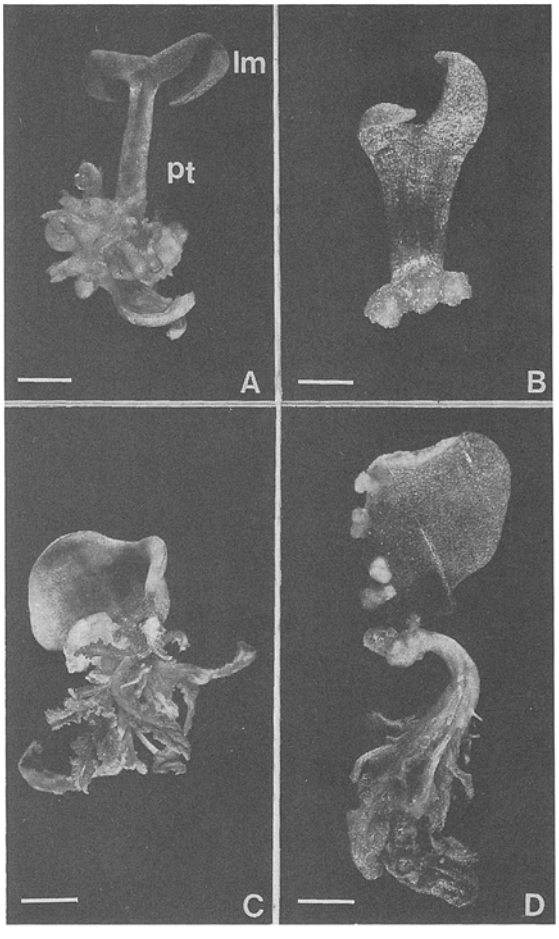

Fig. 2A–D. Regeneration of some of the explants prepared by severing different portions of the cotyledons (see Fig. 1) of *Brassica juncea*. All explants were cultured on MS + 5.0 μM BA for 3 weeks. *A*. The explant E1 (control) has differentiated multiple shoot buds at the cut end of the elongated petiole (lm: lamina; pt: petiole); Bar = 4.0 mm. *B*. The explant E8, in which the entire lamina was removed has expanded and formed a small callus at the basal cut end, but no shoot buds were formed; Bar = 2.0 mm. *C*. The explant E4, which consisted of the upper half of the cotyledon differentiated some shoots at the cut end; Bar = 3.0 mm. *D*. Explant E9, which consisted of a longitudinal half of a cotyledon, developed one shoot at the petiolar cut end and some nodular structures all along the longitudinal cut region of the lamina; Bar = 3.0 mm.

(E8) at different intervals. The presence of the lamina even for one day after culture enhanced the caulogenic as well as the rhizogenic response (Fig. 3). For maximum organogenesis the presence of the lamina was required for 7 days. Beyond this period,

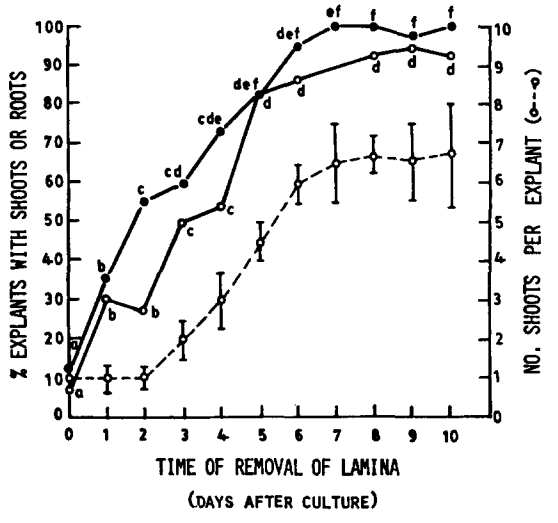

Fig. 3. Effect of severing the lamina at different intervals after the culture of entire cotyledon explants of *Brassica juncea* on shoot (△) and root (▲) differentiation and the number of shoots per responding explant (■) from the petiole. Culture medium: MS + 5.0 μM BA for shoot formation and basal MS for root formation. Growth period: 4 weeks. Frequencies of root formation or shoot formation followed by same letters are not significantly different at $p = 0.05$ (Post-Hoc Multiple Comparisons test). Bars represent standard error of mean.

the presence of the lamina neither improved the frequency of shoot bud formation nor the number of shoots per explant. This was also true for root formation. The addition of 0.05 μM IAA to SIM completely replaced the 'lamina factor', and excised petioles were induced to form shoot buds with a frequency and intensity as high as cotyledon explants on SIM (Table 1).

Table 1. Effects of adding IAA to the shoot induction medium on shoot bud differentiation from cotyledonary petiole explants of *Brassica juncea*. Growth period: 3 weeks

IAA conc. (μM)	Explants with shoots[2] (%)	No. of shoots per responding explant[5]
0 (control I)[1]	86.4[a]	7.2 ± 1.4
0 (control II)[1]	12.6[b]	1.2 ± 0.7
0.01	66.4[a]	1.8 ± 0.9
0.05	90.0[a]	6.5 ± 2.2
0.1	81.8[a]	5.6 ± 1.4

[1] Control I: cotyledon explants cultured on MS + 50 μM BA. Control II: excised petioles cultured on MS + 5.0 μM BA.
[2] Frequencies followed by same letters are not significantly different at $p = 0.05$ (Post Hoc Multiple Comparisons test).
[3] ± represents standard error of mean.

Discussion

Cotyledons play an important role in the normal development of seedlings. A quantitative relationship between the amount of the cotyledonary tissue removed and the extent of growth of the root, hypocotyl, and shoot in embryo cultures of *Vigna sesquipedalis* has been reported by Hotta [7]. He found that the larger the portion of cotyledon severed the greater was the suppression of seedling development. In the embryos of *Cassytha filiformis*, Rangaswamy & Rangan [15] showed that the growth factor(s) for shoot morphogenesis resided in the radicular halves of the cotyledons. To some extent the differentiation of adventitious shoots in citrus [1] and roots in *Vigna radiata* [2] was also influenced by the presence of the cotyledons. These observations suggest that a diffusable promotor of organogenesis emanates from the cotyledons. However, the nature of the 'cotyledon factor' influencing shoot bud differentiation was not determined.

In cotyledon cultures of *B. juncea* the differentiation of shoot buds was restricted to the proximal cut end of the petiole. This was also true for cotyledon cultures of apple [8, 16], and leaf cultures of *Escheveria elegans* [14]. The cotyledons of apple exhibited a gradient of regeneration potentiality, declining towards the distal end. However, in *E. elegans* and *B. juncea* (present study), the excision of the petiole or the proximal portions resulted in total loss of regeneration potential, indicating that the cells competent for shoot formation are restricted to the proximal end [14]. However, unlike the apple and *E. elegans* systems, where removal of the distal half of the cotyledon and leaf, respectively, did not affect the differentiation from the proximal half, in *B. juncea* shoot bud differentiation from proximal end of the petiole, under otherwise optimal conditions (SIM), was dependent on the presence of the lamina. A quantitative relationship was observed between the amount of laminar tissue removed and the degree of decline in shoot bud differentiation.

In our studies the requirement of the lamina for at least 5 days to elicit the near maximum shoot-forming response may be related to the organ induction period. The fact that dark-grown, etiolated

cotyledons do form shoot buds [17], although less frequently, rules out the possibility of photosynthates being the critical factor contributed by the lamina. This then suggests that some regulatory substance(s) emanating from the lamina may be involved in bud differentiation in cotyledon cultures of *B. juncea*. Since the addition of IAA to MS + BA induced shoot bud formation in the cultures of excised pieces of petiole, it can be assumed that the 'lamina factor' is an auxin-like substance. In apple where excision of the distal half of the cotyledon did not influence shoot bud differentiation from the petiole, the culture medium always contained an auxin or coconut milk, a natural source of auxin [16]. The requirement of the lamina for root formation from the cotyledon explants on basal MS medium further supports the idea that the 'lamina factor' may be an auxin-like substance, which in the presence of BA induces shoot bud formation, in keeping with the classical findings of Skoog & Miller [18].

In conclusion, it is evident that the excised cotyledons of *Brassica juncea* form adventitious roots or shoots with high frequencies under fairly simple culture conditions, and that the time and site of differentiation is predictable. Under specified conditions, roots or shoots are formed at the same morphological site within 8–10 days in culture. This system is therefore potentially useful for basic studies on organogenesis, as well as for more applied work, such as selection for somaclonal variation and genetic transformation.

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