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FACTORS AFFECTING HIGH FREQUENCY DIFFERENTIATION OF SHOOTS AND ROOTS FROM COTYLEDON EXPLANTS OF *BRASSICA JUNCEA* (L.) CZERN

KIRAN K. SHARMA[ab], SANT S. BHOJWANI[a] and TREVOR A. THORPE[b]

[a]*Department of Botany, University of Delhi, Delhi 110007 (India) and* [b]*Plant Physiology Research Group, Department of Biological Sciences, University of Calgary, Calgary, Alberta T2N 1N4 (Canada)*

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Studies were undertaken to optimize conditions for high frequency shoot regeneration from excised cotyledons of *Brassica juncea* cv. RIK-81-1. Maximum differentiation of adventitious shoot buds occurred when the explants derived from 5-day-old seedlings were cultured on Murashige and Skoog medium (MS) containing 5 μM N^6-benzyladenine (BA). On MS alone only roots were formed. Shoot or root formation was restricted to 1–2 mm of tissue at the cut end of the petiole. Organogenesis occured only if the proximal cut end of the petiole was in contact with the medium. The lamina did not exhibit organogenesis. Cotyledons cultured for up to 3 days on root induction medium (MS), still retained their full potential to form shoots upon transfer to MS + BA. With longer incubation on MS medium the shoot-forming capacity of the explants declined, and after 8 days it was completely lost. When applied through the agar medium, BA (5 μM) was required for at least 7 days for shoot bud induction. The possible usefulness of *B. juncea* cotyledons as an experimental system for detailed studies of morphogenesis and genetic transformation is discussed.

Key words: *Brassica juncea*; brown mustard; cotyledon culture; regeneration; organogenesis; tissue culture

Introduction

Regeneration of mature plants from undifferentiated tissues is central to the application of biotechnology to crop improvement. Although morphogenesis and regeneration in tissue culture have been the subjects of intense investigations for more than two decades, the nature and control of organ differentiation remains largely unexplained [1]. Consequently, information concerning induction of plant regeneration in tissue culture results largely from empirical studies. Understanding the process of differentiation from explants may prove rewarding in the systematic manipulation of conditions for controlled organogenic differentiation in callus and cell cultures. The basic attributes of an ideal experimental system for such studies would be that it forms shoots with high frequency on a minimal medium, and the time and site of differentiation are predictable. Moreover, the cells undergoing differentiation should be easily removable and available in large quantities for detailed biochemical studies [1]. Although attempts have been made to develop such a system, most attempts have so far fallen short of this goal.

In the present study we report that excised cotyledons of *Brassica juncea* can be made to reproducibly form roots or shoots under very simple culture conditions, and that the organogenic differentiation is restricted to the peripheral cells at the proximal cut end of the petiole.

Materials and Methods

Preparation of explants

The seeds of *B. juncea* (L.) Czern. cv. RIK-81-1 were obtained through the courtesy of Mr. S.D. Dubey, Regional Research Station, Indian Agricultural Research Institute (I.A.R.I.), Kanpur. Aseptic seedlings served as the source of cotyledon explants. To raise seedlings, seeds were given a quick rinse in 90% ethanol and then sterilized in 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 MS [2] basal medium.

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Individual cotyledons were excised from seedlings about 1 mm below the cotyledonary node. Unless otherwise stated, cotyledons from 5-day-old seedlings were used. The cotyledon explants measured 4–6 mm, and contained a bilobed lamina and a short (about 2 mm long) petiole. Hereafter, the end of the cotyledon close to the embryonal axis is called the 'proximal end' while the one away from the embryonal axis is the 'distal end'. Similarly, the surface of the lamina facing the embryonal axis is referred to as the 'adaxial surface' and the other surface is the 'abaxial surface'. Normally, the cotyledons were planted with their abaxial surface in contact with the medium and the proximal cut end embedded in the medium.

Culture medium

All the experiments were conducted using MS basal medium. It was variously modified in different experiments. The supplements tested include N^6-benzyladenine (BA), kinetin, 2-isopentenyl adenine (2-ip), zeatin, and α-naphthaleneacetic acid (NAA). Routinely, 15 ml of the molten medium containing all the constituents was poured into 150 mm × 25 mm rimless Corning tubes. The tubes were plugged with non-absorbant cotton wrapped in one layer of cheesecloth, and sterilized by autoclaving at 1.06 kg cm^2 pressure and 121°C temperature for 15 min. The pH of the medium was adjusted to 5.8 prior to autoclaving. Zeatin was filter-sterilized and added to the autoclaved medium before solidification.

Culture conditions

All cultures were maintained at 25 ± 2°C under continuous light of 80 μE m^{-2} s^{-1} irradiance provided by fluorescent lamps (TL 40 W/54 cool daylight).

Observations of cultures and presentation of results

Usually 24 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. Wherever possible, the effects of different treatments were quantified on the basis of per cent cultures showing the response and the degree of response per culture. The data pertaining to frequencies of shoot bud differentiation were subjected to chi square test for homogeneity of proportions and significant treatment differences selected by Post Hoc Multiple Comparisons test [3]. Within each treatment, numbers followed by same letters are not significantly different at the $P = 0.05$ level.

Results

Initial experiments used cotyledon explants derived from 7-day-old seedlings of *B. juncea* in which the MS medium was supplemented with different concentrations of BA (0.5,1.0, 5.0, 10.0, 50.0 μM) and NAA (0.1, 0.5, 1.0, 5.0, 10.0 μM) individually and in different combinations in a Latin square design. In the absence of any growth regulator only roots were formed without any apparent sign of callus formation. Addition of BA suppressed root formation and induced shoot bud differentiation. The highest frequency of shoot bud differentiation occurred with 5.0 μM BA alone. The addition of NAA in conjunction with BA reduced the caulogenic response and promoted callus formation and/or rooting. Callus growth was best in the presence of 5.0 μM BA and 0.5 μM NAA. The callus was semi-friable and did not regenerate plants on subculture despite several attempts. Further attempts were mainly confined to optimizing conditions for high frequency shoot bud differentiation from the cotyledon explants.

Age of cotyledon donor seedlings

In order to study how the changing physiology of the cotyledons affects their regeneration potential, cotyledons were taken from 1–10-day-old seedlings and cultured on MS + BA (5.0 μM). The percentage of cultures with shoot bud differentiation increased with age of seedlings, reaching a maximum at 5 days (Fig. 1). Thereafter, the response sharply declined, and the cotyledons from 8-day-old seedlings showed negligible regeneration. The number of shoots per explant also sharply declined after the 5th day. The cotyledons derived from 6-day-old and older seedlings exhibited yellowing of the lamina after 18–20 days of culture, and the shoots differentiating from them did not grow as vigorously as those formed by cotyledons from 1–5-day-old seedlings. In the latter case the cotyledons appeared green up to

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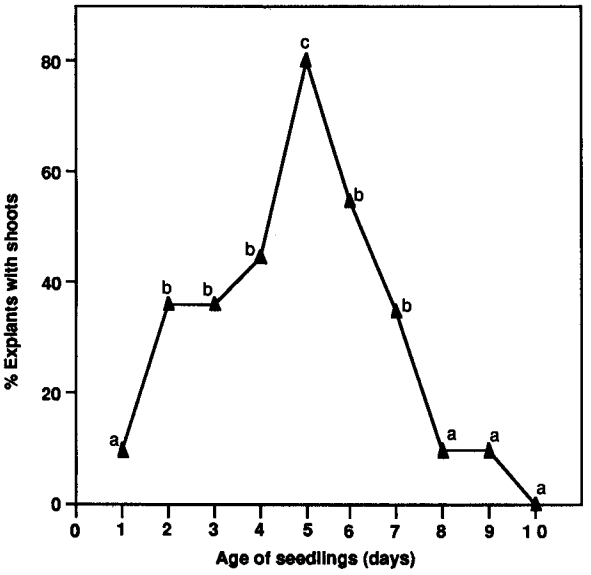

Fig. 1. Effect of the age of the donor seedlings of *B. juncea* on shoot bud differentiation in excised cotyledons. Data points indicated by similar letters are not significantly different at $P = 0.05$ (Post Hoc Multiple Comparisons test). Culture medium: MS + 5.0 μM BA . Growth period: 3 weeks.

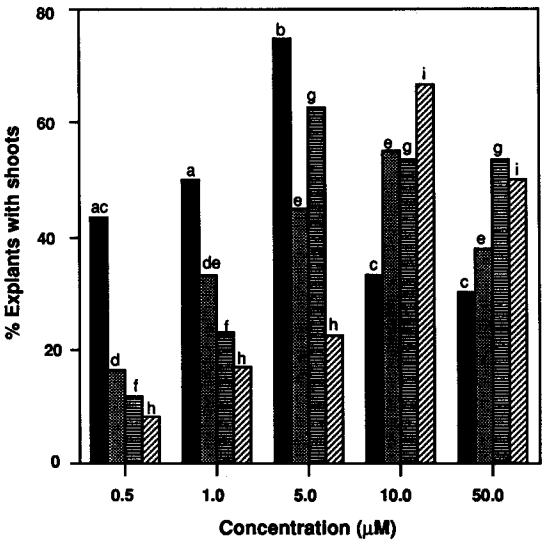

Fig. 2. Effect of BA (■), zeatin (▩), 2-ip (▤), and kinetin (▨) on shoot bud differentiation in excised cotyledons of *B. juncea*. Within each treatment, bars indicated by similar letters are not significantly different at $P = 0.05$ (Post Hoc Multiple Comparisons test). Basal medium: MS; Growth period: 3 weeks.

at least 28 days after culture initiation. Therefore, cotyledons from 5-day-old seedlings were used in all further studies.

Efficacy of various cytokinins

The effect of BA on shoot bud differentiation was compared with three other cytokinins viz., kinetin, 2-ip and zeatin. All the cytokinins, including BA were added to the MS medium at five different concentrations (0.5, 1.0, 5.0, 10.0 and 50.0 μM). In the medium lacking a cytokinin the explants enlarged, and after 5–7 days differentiated roots in 90% of the cultures. In older cultures the roots were branched.

All the cytokinins tested induced shoot bud differentiation but with different frequencies (Fig. 2). The optimum level of BA and 2-ip was 5.0 μM and that of kinetin and zeatin 10.0 μM. Comparing their optimum levels, BA was the most effective cytokinin in terms of the number of cultures forming shoots and the number of shoots per explant. Whereas, 5.0 μM BA induced multiple shoot formation (about 8 shoots), with the other three cytokinins generally a solitary shoot developed.

On MS + BA (5.0 μM), within 3 days the lamina enlarged almost 2–3 times its original size and the petiole elongated considerably. Shoot buds appeared 10–12 days after culture initiation and were restricted to the proximal cut end of the petiole. In older cultures a nodular, hard callus also developed at the base of the adventitious shoots.

Pulse treatment of BA

Since BA (5.0 μM) was the most effective cytokinin for shoot formation, the minimum duration for the BA treatment was assessed. Cotyledons were cultured on MS + BA (5.0 μM) for different periods before transferring them to MS basal medium or vice versa and their organogenic responses were recorded (Fig. 3).

A 1-day exposure to BA neither induced shoot bud formation nor suppressed rooting (Fig. 3A). Maintaining the cotyledons on BA-containing medium for 3–5 days induced shoots only in a few cultures. For maximum shoot formation BA was required for at least 11 days. Thereafter, the number of cultures forming shoots did not increase significantly, although the number of shoots per explant reached its maximum (up to 8 shoots) on the

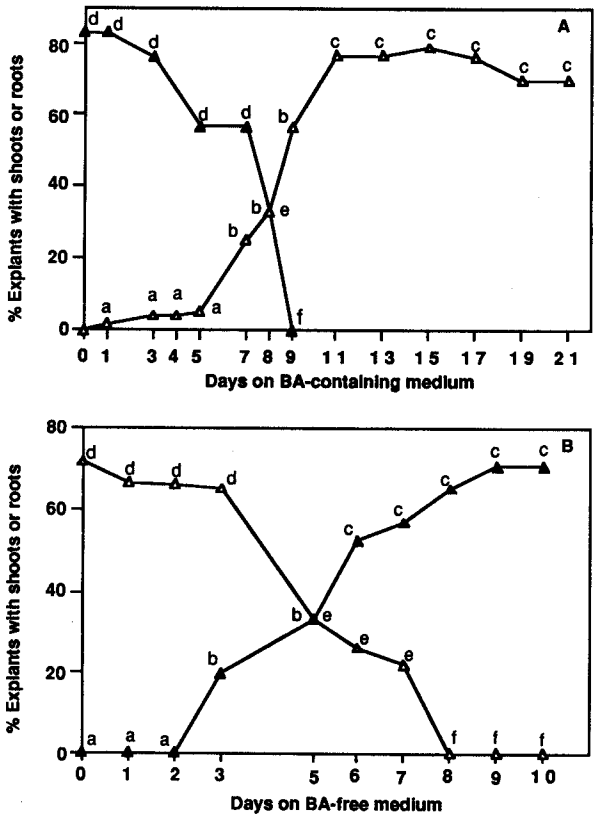

Fig. 3. Effect of the duration of BA application on shoot (△) and root (▲) differentiation in excised cotyledons of *B. juncea*. The explants were cultured on MS + 5.0 μM BA for various periods and then transferred to MS alone (A) or the explants were cultured on the MS medium for various periods and then transferred to MS + 5.0 μM BA (B). Within each treatment, data points indicated by similar letters are not significantly different at $P = 0.05$ (Post Hoc Multiple Comparisons test). Growth period: 3 weeks.

13th day of treatment. Rooting response was almost inversely proportional to the length of the BA treatment, being completely suppressed by 9 days. Application of BA in liquid form even for 24 h proved inhibitory for shoot bud differentiation (data not presented).

When the cotyledons were grown on MS medium for various periods before transferring them to MS + 5.0 μM BA, the explants retained their full potential to respond to the BA treatment for shoot formation up to 3 days after excision (Fig. 3B). Thereafter, the shoot-forming potential declined sharply, and no shoots were formed if the time between excision of the cotyledons and BA application was longer than 7 days. In contrast, root differentiation occurred if the excised cotyledons did not receive BA application for at least 3 days, and this response steadily increased with further delay in BA application. Interestingly, only rarely were both shoots and roots formed on the same explant.

Nutrients

Excised cotyledons cultured on shoot-forming medium were green and exhibited considerable expansion before the formation of shoots. Therefore, the requirement for exogenous carbohydrate was examined. The excised cotyledons were cultured on MS + BA (5.0 μM) without or with sucrose or glucose at different concentrations ranging 0–8%.

Shoot bud formation was completely inhibited on the medium devoid of a carbohydrate. With 1% sucrose only about 5% of the cultures formed 1 or 2 shoots which turned brown without much further growth (Fig. 4). There was a dramatic increase

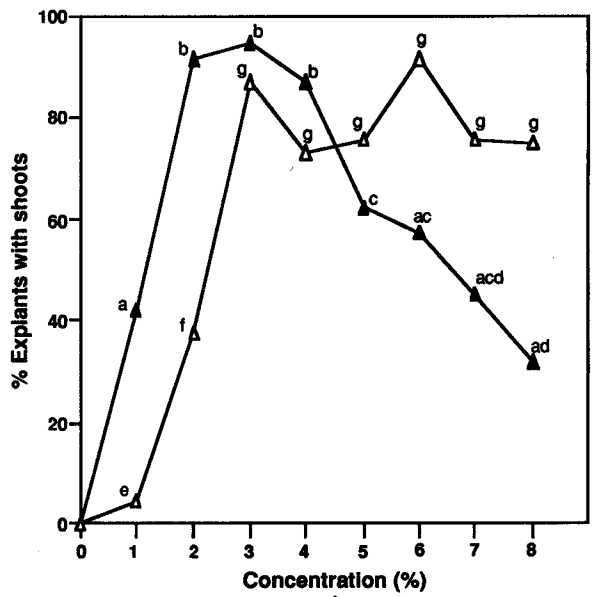

Fig. 4. Effect of sucrose (△) and glucose (▲) on shoot bud differentiation in excised cotyledons of *B. juncea*. Within each treatment, data points indicated by similar letters are not significantly different at $P = 0.05$ (Post Hoc Multiple Comparisons test). Culture medium: MS(-sucrose) + 5.0 μM BA; Growth period: 3 weeks.

in the frequency of shoot bud differentiation with increase in sucrose concentration up to 3%. At higher levels of sucrose, shoot bud formation was accompanied by callus formation at the base of the shoots. Maximum callusing occurred with 5% sucrose. At 6% sucrose, which was as good as 3% in terms of the number of shoots formed, most of the shoots appeared to differentiate from the callus. The shoots formed in the presence of sucrose at levels higher than 3% were deep green in color and healthy but remained stunted.

Shoot bud differentiation also occurred when glucose was used instead of sucrose (Fig. 4). With regard to the frequency of shoot bud differentiation, glucose was significantly better than sucrose at corresponding levels up to 4%. In terms of the number of explants which formed shoots 2% glucose was as effective as 3% sucrose. However, the shoots formed with 3% sucrose were distinctly healthier than with 2% glucose. At concentrations higher than 4%, glucose was more toxic than sucrose.

In a separate set of experiments, major salts, minor salts and organics from the MS medium were eliminated individually and in different combinations from shoot induction medium. The complete absence of any one category of the nutrients resulted in the total lack of shoot bud differentiation. Even the expansion of cotyledons on different media was extremely poor. Deletion of individual major salts from the MS medium also caused a reduction in the percentage of cultures forming shoots, as well as the number of shoots per responding explant (data not presented).

Delayed application of nutrients

To determine whether the MS nutrients are required throughout the culture period or only during the post-inductive phase of shoot development, an experiment was done in which cotyledons were cultured on agar-water-BA (5.0 μM) medium for various periods before transferring them to the MS + BA medium. The absence of MS nutrients during the initial 3–4 days caused a non-significant decrease in the number of explants forming shoot buds (Fig. 5). However, a further delay in the application of nutrients drastically reduced the frequency of responding cultures. Nevertheless, the number of shoots per responding explant was not affected.

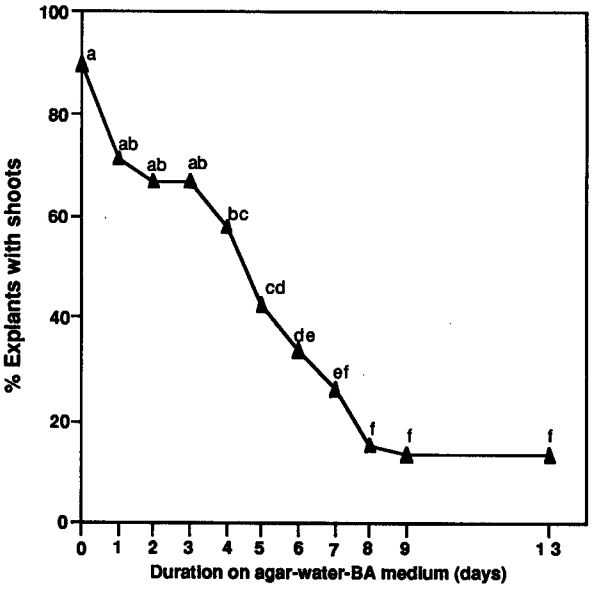

Fig. 5. Effect of delayed application of MS nutrients on shoot bud differentiation in excised cotyledons of *B. juncea*. The explants were cultured on agar-water-BA medium for 1–13 days and then transferred to MS + 5.0 μM BA. Data points indicated by similar letters are not significantly different at $P = 0.05$ (Post Hoc Multiple Comparisons test). Total growth period: 3 weeks.

Orientation of the explant

A general observation in cotyledon cultures during shoot formation was that the margins of the expanding cotyledons curled towards the abaxial side of the explant lifting the cut end of the petiole away from the medium. If the petiole lost contact with the medium within 3 days of culture initiation, it did not form shoot buds. This phenomenon was considered to be the main cause of considerable variation observed in the frequencies of shoot differentiation under specified culture conditions. An experiment was, therefore, conducted to study the effect of orientation of the explant on shoot bud differentiation.

Cotyledons were planted on MS + BA (5.0 μM) medium in six different orientations and their shoot forming response was compared after 3 weeks. Shoot bud differentiation only occurred in these treatments where the petiole was kept in contact with the medium. In this treatment, the explants were planted with the baxial surface in contact with the medium and the petiole embedded in the me-

dium. In a treatment where the explants were planted with adaxial surface in contact with medium and the petiole curved away, the response was extremely low, probably because in this position the petiole could not be easily bent towards the medium without causing injury.

Discussion

The classical findings of Skoog and Miller [4] that organogenesis in tissue cultures is governed by the balance of auxin and cytokinin in the medium cannot be demonstrated universally due to the explant sensitivity or the original content of endogenous growth regulators. Dunwell [5] reported that the three species of *Brassica* viz., *B. campestris*, *B. napus* and *B. oleracea* differed in their response in culture to particular combinations of auxin and cytokinin. In all the previous reports dealing with cotyledon cultures of *B. juncea*, an auxin and a cytokinin were used for shoot bud differentiation [6–8]. George and Rao [6] observed that the maximum regeneration with BA (0.2 mg l^{-1}) alone was 17% while with BA and NAA 95% cultures formed shoot buds. In contrast to these reports, in the present study, a cytokinin alone was found optimum for the induction and subsequent growth of shoot buds in cotyledon cultures of cultivar RIK-81-1 of *B. juncea*.

The presence or absence of a cytokinin in the medium determines the nature of organogenic differentiation from cotyledon explants of *B. juncea* (present study). Whereas on MS alone (root inducing medium; RIM) only roots were formed, in the presence of BA only shoot bud differentiation occurred. If cotyledons are cultured without BA for a period of 7 days the competent cells at the petiolar cut end become fully determined to form roots, and their subsequent transfer to BA-containing medium did not alter the destiny of these cells. Similarly, the minimum time required in the presence of BA for any shoot differentiation was 3 days, and within 7 days cells become determined for shoot formation. In *Convolvulus arvensis* the determination for shoot formation occurs only after 10 days on shoot-forming medium. Such explants continue to form shoots even after transfer to root-forming conditions [9]. The cotyledons of *Pinus radiata* suffer irreversible loss of shoot forming capacity if they do not receive the inductive cytokinin treatment during the initial 24 h in culture [10].

In cotyledon cultures of *B. juncea*, the absence of MS nutrients during the initial 3 days of BA treatment did not affect the frequency of shoot bud differentiation but their absence beyond 5 days drastically reduced shoot formation. These observations and those of von Arnold and Eriksson [11] and von Arnold [12] with the embryos of *Picea abies* and Tanimoto and Harada [13] with *Torenia* stem segments suggest that exogenous nutrients are not required for the initial stages of shoot bud induction but are essential for subsequent development of shoot buds. Consistently high frequency shoot differentiation was observed in cotyledon cultures of *Brassica juncea*. Shoot/root formation was restricted to the proximal cut end of the petiole and the physical contact of the petiole with the culture medium was essential to elicit a complete response. It is, therefore, not surprising that Lazzeri and Dunwell [14] and Murata and Orton [8], who used laminar discs or segments found that cotyledon explants of *B. oleracea* showed poor and sporadic regeneration. In our laboratory the cotyledon explants of *B. oleracea* prepared as in the present study also proved highly regenerative [15]. Also there was an optimum age (5 days) for selecting the cotyledon explants, as found in other studies, e.g., *Pinus radiata* [16]. Moreover, organ differentiation from cotyledons occurs only if the site of organogenesis, i.e., cut end of the petiole maintains a contact with the medium for at least 3–5 days from culture initiation. This suggests that the absence or presence of cytokinin may be effective only when it is directly taken up by the 'target cells' at the cut end or the surface in contact with the culture medium.

Our knowledge of the physiology and biochemistry of the organ formation process is still limited due to the lack of a truely suitable experimental system (see Introduction). Excised cotyledons of *B. juncea* may be ideal for such studies. They form adventitious shoots or roots with high frequencies under fairly simple culture conditions. Under specified conditions, roots and shoots are formed at the same morphological site but from different cells (manuscript in preparation) within 8–10 days. Hence, cells which are synchronously undergoing dedifferentiation followed by differentiation de novo should be

readily available in large quantities for detailed biochemical studies. In addition, this cotyledon system has been efficiently used recently for *Agrobacterium*-mediated gene-transfer in *B. napus* with high transformation frequencies [17]. The shoots regenerated from the cotyledons of *B. juncea* appear morphologically normal and after rooting and transplantation to the greenhouse produce flowers and set seeds. The plants thus obtained are also proving to be a rich source of somaclonal variants [18; Arora and Bhojwani, unpublished].

Acknowledgements

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