Research Communication

Benzyladenine-preconditioning in Germinating Mungbean Seedlings Stimulates Axillary Buds in Cotyledonal Nodes Resulting in Multiple Shoot Regeneration

Renato A. Avenido1,2 and Kazumi Hattori1)*

1) Laboratory of Plant Genetics and Breeding, Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya, Aichi 464-8601, Japan
2) Institute of Plant Breeding, College of Agriculture, University of the Philippines Los Baños, College, Laguna 4031, The Philippines

Tissue culture and histological studies were done to ascertain the effects of benzyladenine (BA)-preconditioning on direct shoot regeneration from cotyledonal node explants of mungbean. The highest direct shoot regeneration (93.3%) of double-sided shoot initiation (65.0%) and average number of shoots per explant (2.7 shoots) were obtained when Pag-asa 7 explants are excised from seedlings germinated in basal medium (BM) consisting of MS salts and B5 vitamins with 1.0 mg l⁻¹ BA, and subsequently cultured in fresh medium of the same composition. Histological inspection of sections from germinating seeds in BM and BM + 1.0 mg l⁻¹ BA sampled at 1 to 4 days from germination revealed bigger and developmentally more advanced axillary shoots in BA-preconditioned seedlings, a built-in advantage which could attest to the improved regenerability in BA-treated seedling explants over the control (BM only). During shoot initiation from explants, two treatments namely doubling of the concentration BA to 2.0 mg l⁻¹ and the addition of a non-ionic surfactant, Pluronic F-68 did not further increase the responses, but gave results comparable with those obtained using BM + 1.0 mg l⁻¹ BA alone. Conversely, a BA-thidiazuron (TDZ) combination (each at 1.0 mg l⁻¹) when added to BM significantly reduced shoot induction to only 48.3% possibly due to increased callusing at the nodes in 55% of the explants. The importance and applicability of the regeneration system in tissue culture and/or genetic manipulations of mungbean and other Asiatic Vigna species is discussed.

Key Words: Vigna radiata L. Wilczek, axillary bud, BA-preconditioning, cotyledonal node explant, histology, tissue culture.

Introduction

Mungbean (Vigna radiata L. Wilczek) is the most popular and widely cultivated pulse among the Asiatic Vigna (subgenus Ceratotropis) species the world over. Due to its importance in human nutrition, biotechnology in conjunction with conventional breeding techniques is seen to play a pivotal role in genetic improvement programs. However, progress in this field has been stalled by the lack of reproducible plant regeneration systems from cell and tissue cultures. Despite the strong recalcitrance from callus, cell or protoplast cultures, direct and multiple shoot formation has been achieved using cotyledonal node explants from in vitro-grown seedlings of mungbean (Avenido and Hautea 1990, Avenido et al. 1991, Gulati and Jaiwal 1994, Avenido and Hattori 1999). The application of benzyladenine (BA) during seed germination (i.e. BA-preconditioning) increased the shoot regeneration efficiency in the cotyledonal node explants in these studies. However, no histological data on the comparison of axillary shoot development during seed germination as affected by BA-preconditioning is available.

In a related study, direct shoot induction from the cotyledonal node explant was found applicable to all the epigeal and allotetraploid Asiatic Vigna species (Avenido et al. 2001). Hence, further improvement (i.e. efficient multiple shoot regeneration from both axils, that is, double-sided response) and clearer understanding of axillary shoot development in mungbean explants may also be beneficial to tissue culture and/or genetic manipulation (i.e. gene transfer) of other important epigeal Vigna species within the subgenus Ceratotropis.

In this study, we examined the effects of factors involving cytokinin treatments (i.e. BA-preconditioning, BA-thidiazuron [TDZ] combination) and incorporation of a non-ionic surfactant, Pluronic F-68 (PF-68) applied to mungbean cotyledonal node explants to further improve shoot regeneration efficiency. For the first time, histological observations on axillary shoot development of in vitro germinated seedlings in hormone-free and BA-supplemented medium were made in order to clearly establish the beneficial effects of BA-preconditioning at the pre-transportation stage in relation to shoot regeneration from cotyledonal node explants after subsequent culture.

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*Corresponding author (e-mail: kazuhatt@agr.nagoya-u.ac.jp)
Materials and Methods

Plant materials, explant preparation and shoot initiation
Mature seeds of green-seeded mungbean (Vigna radiata L. WIlczek) cv Pag-asa 7 were surface sterilized in 70% (v/v) ethanol (1 minute), followed by immersion in 50% (v/v) Antiformin (sodium hypochlorite solution [5.0% a.i.]) for 30 minutes and finally rinsed 5 times with sterile distilled water. At seed germination, the basal medium (BM) consisting of MS salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al. 1968) and 3.0% (w/v) sucrose medium alone and BM with the addition of 1.0 mg l⁻¹ BA (BM + 1.0 mg l⁻¹ BA) were used. Using 4-day-old seedlings, cotyledonal node explants were prepared and cultured aseptically according to Avenido and Hattori (1999). For subsequent shoot initiation from explants, different media consisting of BM with BA (1.0 and 2.0 mg l⁻¹) alone and with further addition of either the non-ionic surfactant, Pluronic F-68 (PF-68) at 0.01%, w/v or thidiazuron (TDZ) at 1.0 mg l⁻¹ were used. The six different media treatments for seed germination and subsequent explantation for shoot initiation were as follows: T1) BM : BM; T2) BM : BM + 1.0 mg l⁻¹ BA; T3) BM + 1.0 mg l⁻¹ BA : BM + 1.0 mg l⁻¹ BA; T4) BM + 1.0 mg l⁻¹ BA : BM + 2.0 mg l⁻¹ BA; T5) BM + 1.0 mg l⁻¹ BA : BM + 1.0 mg l⁻¹ BA plus TDZ and T6) BM + 1.0 mg l⁻¹ BA : BM + 1.0 mg l⁻¹ BA plus PF-68 (see Table 1). All media were adjusted to pH 5.8 before addition of agar (0.9% w/v, INA BA-30), dispensed into 5.7 × 10.2-cm bottles in 40-ml aliquots and sterilized at 121°C for 20 minutes. Inoculated culture bottles were covered with polypropylene plastic sheets and incubated at 28°C under continuous light (25 µmol m⁻² s⁻¹).

Replication, data collection and data analysis
Five explants were inoculated per bottle for shoot initiation and four bottles containing a total of 20 cotyledonal node explants represented each treatment. The experiment was replicated thrice. Data on percentage shoot regeneration were obtained at 4, 8 and 28 days after explantation while the average number of shoots was collected from 28-day-old cultures. Double-sided shoot regeneration was used to refer to shoot initiation event wherein shoots arise bilaterally from both axes found above the cotyledon detachments (i.e. both sides of cotyledonal node). In scoring for the number of shoots, each shoot consisted of apical meristem and leaves whose length from base to meristem was at least 4.0 mm and smaller shoots were not counted. When necessary, percentage data were transformed by the arcsine method prior to analysis of variance (ANOVA). Differences among means were analyzed using the Duncan’s Multiple Range Test (DMRT) at the 5% level.

Histology and light microscopy
In this experiment, thirty germinating seedlings cultured in BM alone (control) and BM + 1.0 mg l⁻¹ BA were sampled separately after 1, 2, 3 and 4 days of incubation. Samples were fixed in FAA (formalin, acetic acid, 70% ethanol at 5 : 5 : 90 ratio), dehydrated in a graded acetone series (30, 50, 70, 90, 95, 100%, v/v) at 30-minute intervals and then treated with propylene oxide for 24 hours. With daily changes of resin containing glycol methacyrlate as the main component, samples were vacuum-infiltrated for a week prior to embedding. Tissue blocks were trimmed and sectioned longitudinally to 6 µm on a rotary microtome. Sections were stained with Toluidine Blue (0.05%, w/v; 2 minutes) and observed under a light microscope. From these, the meristem diameter was measured at the base of axillary shoots in at least seven samples per treatment.

Results and Discussion

Cytokinin requirements for direct shoot induction from cotyledonal nodes
Among the cytokinins, BA is the most frequently and most successful plant growth regulator used for micropropagation (Thomas and Blakesley 1987). It is also the most commonly used for direct induction of shoots from tissue cultured-cotyledonal node explants of various grain legumes (Shiva Prakash et al. 1994, Polisetty et al. 1997), leguminous tree (Dewan et al. 1992, Pradhan et al. 1998) and other industrial tree (Bhuyan et al. 1997, Mao et al. 2000) species. In mungbean cv Pag-asa 7, addition of BA at 1.0 mg l⁻¹ to MS-B5 basal medium (BM + 1.0 mg l⁻¹ BA) used during seed germination (i.e. BA-preconditioning) of explant sources is required for early, double-sided and multiple induction of shoots from the axes of the cotyledonal node explants (Table 1). Percentage of shoot induction in 4- and 8-day-old cultures were consistently higher when BA was used during seed germination and shoot initiation from explants (T3) with 90.0 and 93.3% as compared when no BA was used (T1) at 33.3 and 63.3% or when BA was added only during shoot initiation (T2) at 28.3 and 58.3%. Similarly, T3 induced the highest double-sided shoot initiation of 65.0% as compared with that of T1 and T2 with only 15.0 and 20.0% responses, respectively. In Vigna angularis, BA was also found to be the most effective in inducing adventitious shoot formation from the hypocotyls of cotyledonal node explants when used in both seed germination and shoot induction media at 1.0 mg l⁻¹ (Avenido and Hattori 2000). The beneficial effects of using BA during seed germination (BA-preconditioning) to increase shoot regeneration efficiency among the explants were applied earlier in Vigna radiata (Avenido and Hautea 1990, Avenido et al. 1991, Narciso et al. 1997) and other legumes such as Glycine max (Thome et al. 1995, Wright et al. 1986), Arachis hypogea (Daimon and Mii 1991), Phaseolus spp. (Malik and Saxena 1992, Santalla et al. 1998) and Cajanus cajan (Shiva Prakash et al. 1994). However, a comparison of the BA treatments during seed germination was not presented in these reports. In Murraya koenigii, significantly early and efficient seed germination resulting in subsequent large-scale shoot production was dependent on the addition of 5.0
mg l⁻¹ BA in seed germination medium (Bhuyan et al. 1997).

Further addition of the non-ionic surfactant PF-68 at 0.01 % during shoot initiation of BA-preconditioned explants (T6) resulted in responses comparable to that of using BM + 1.0 mg l⁻¹ BA only (T3) (Table 1). In Vigna angularis, PF-68 also failed to further improve the adventitious shoot formation from seedling hypocotyl explants (Avenido and Hattori 2000). However, significant improvement in shoot production has been reported from stem and leaf explants of Populus spp. (Jordan-Costache et al. 1995) and petiole and cotyledon explants of Corchorus capsularis (Khatun et al. 1993) brought about by addition of 0.001 to 0.5 % Pluronic F-68 in the regeneration media. This effect was attributed partly to increased permeability of plasma membranes to nutrients and/or growth regulators.

Doubling the BA concentration to 2.0 mg l⁻¹ (T4) during shoot initiation from explants did not further increase the shoot regeneration response. In earlier studies on mungbean (Gulati and Jawial 1990, 1992, 1994) and other grain legumes (Wright et al. 1986, Shiva Prakash et al. 1994, Avenido and Hattori 2000), BA at 1.0 to 2.0 mg l⁻¹ were also found optimum for shoot regeneration. Likewise, further addition of 1.0 mg l⁻¹ TDZ to BM + 1.0 mg l⁻¹ BA medium (T5) significantly reduced the percentage of shoot initiation (33.3 and 48.3 %), double-sided response (10.0 %) and promoted the highest incidence of callusing at the nodes (55.0 %) which even prevented initiated shoots to elongate (Table 1). A similar negative effect of TDZ was observed earlier in Vigna angularis when the cotyledonal node explant formed prolific callus instead of adventitious shoots (Avenido and Hattori 2000). On the contrary, a combination of BA and TDZ was found to be highly beneficial for multiple shoot regeneration from cotyledonal nodes of the woody species, Fraxinus pennsylvanica (Kim et al. 1997) and the annual grain legume, Vicia faba (Khalafalla and Hattori 1999).

In terms of average number of shoots at 28 days after culture, doubling of BA concentration (T4) or further addition of PF-68 (T6) did not significantly increase the number of shoots when compared with that of using BM + 1.0 mg l⁻¹ BA only. However, three treatments (T3, T4 and T6) gave significantly higher responses (2.63 to 2.79 shoots) than the other treatments (1.3 to 2.0 shoots) (Table 1). In an earlier study, the applicability of using BM + 1.0 mg l⁻¹ BA during seed germination and shoot initiation (T3 in this report) was demonstrated in five other genotypes of mungbean (Avenido and Hattori 1999). Avenido and Hattori (1999) also reported that biweekly subculture and removal of basal callus from the hypocotyls of the regenerating explants significantly increased shoot production in mungbean and other genotypes of seven other Asiatic Vigna species or subspecies.

**Benzyladenine-preconditioning stimulates axillary bud development in mungbean seedlings**

Histological studies on germinating Pag-asa 7 seedlings from day 1 to day 4 revealed bigger and developmentally more advanced axillary shoots from BA-preconditioned seedlings cultured in vitro over that of BM alone (Fig. 1). At day 1, the average meristem diameter measured at the base was 72.9 ± 5.7 and 90.0 ± 16.8 μm for the control and BA-preconditioned seedlings, respectively. At day 2, meristem diameter was also bigger (106.9 ± 18.7 μm) in BA-treated shoots than the control (85.7 ± 14.3 μm). At day 3, meristem diameter in BA-treated seedlings significantly increased to 147.5 ± 11.3 μm as compared to the control (86.2 ± 10.6 μm). This difference in meristem diameter was even more evident at day 4 with meristems from the control measuring 98.6 ± 14.6 μm as compared with BA-treated (162.9 ± 36.2 μm) seedlings. Likewise, there was a corresponding increase in the circumference of the hypocotyl giving the BA-treated seedlings a fatter appearance. These observations attest to the beneficial effects of BA during seed germination for increasing efficiency in direct shoot

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**Table 1.** Effects of BA-preconditioning and different combinations of BA, TDZ and Pluronic F-68 on the frequency of shoot induction and average number of shoots from cotyledonal node explants of mungbean (V. radiata L. Wilczek) cv Pag-asa 7 at 4, 8 and 28 days from explantation

| Treatment/media | % Shoot induction | % Explants with callused nodes | Average number of shoots at 28 days
<table>
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<tr>
<td>Seed Germination : Explant Culture</td>
<td>at day 4 total at day 8</td>
<td>at day 8</td>
<td>at day 8</td>
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<td>-----------------</td>
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<tr>
<td>T1. BM</td>
<td>: BM</td>
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</tr>
<tr>
<td>T2. BM</td>
<td>: BM + 1.0 BA</td>
<td>33.3 b</td>
<td>63.3 b</td>
</tr>
<tr>
<td>T3. BM + 1.0 BA</td>
<td>: BM + 1.0 BA</td>
<td>28.3 b</td>
<td>58.3 b</td>
</tr>
<tr>
<td>T4. BM + 1.0 BA</td>
<td>: BM + 1.0 BA</td>
<td>90.0 a</td>
<td>93.3 a</td>
</tr>
<tr>
<td>T5. BM + 1.0 BA</td>
<td>: BM + 1.0 BA + 1.0 TDZ</td>
<td>85.0 a</td>
<td>90.0 a</td>
</tr>
<tr>
<td>T6. BM + 1.0 BA</td>
<td>: BM + 1.0 BA + PF-68</td>
<td>33.3 b</td>
<td>48.3 b</td>
</tr>
<tr>
<td>T7. BM + 1.0 BA</td>
<td>: BM + 1.0 BA + PF-68</td>
<td>86.7 a</td>
<td>93.3 a</td>
</tr>
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1) All media consisted of MS salts, B5 vitamins and 30 g l⁻¹ sucrose basal medium (BM) with the addition of two cytokinins (BA, TDZ) at 1.0 mg l⁻¹ (unless otherwise specified) and Pluronic F-68 at 0.01 % (w/v).

2) Treatment means are collected from 3 independent experiments, each with 20 replicates.

3) Counted from all explants with shoots induced (in three replications).

Means within a column with different letters are significantly different by DMRT.
nc = shoot buds were not counted.
regeneration, a procedure currently being employed in many plant regeneration systems. In soybean, BA has been reported to activate the presence of totipotent cells at the cotyledonary node region (Cheng et al. 1980) leading to shoot formation after in vitro culture (Wright et al. 1986). In an earlier study using water-imbibed seeds subsequently cultured in a medium similar to BM + 1.0 mg l⁻¹ BA, nodules with a distinct tunica layer and leaf primordia were observed from the basal adaxial side of the petiolar residue after 60 hours (Mendoza et al. 1993). This regeneration site corresponded with the cotyledon-stem junction or the area just above the detachment area once the cotyledons are removed. In a related study, the axillary buds in the cotyledonary nodes were found already fully-developed in the epigeal V. radiata and hypogeal but allotetraploid V. glabriscens 4 days after germination in BM + 1.0 mg l⁻¹ BA (Avenido et al. 2001). Hence, the capacity of the explants to regenerate shoots was already predetermined at the
time of explantation and is realized under favorable culture conditions.

Previous histological examination in V. radiata (Mendoza et al. 1993, Gulati and Jaiwal 1994) and in Pismus sativum (Jackson and Hobbs 1990) revealed that the shoots developed in a manner consistent with axillary shoot proliferation. They also noted that shoot differentiation occurred directly from epidermal and sub-epidermal cells without callus formation at the node. The same origin of shoot buds was also reported in Phaseolus vulgaris (McClean and Graffen 1989) and Phaseolus species (Malik and Saxena 1992).

In conclusion, direct shoot induction from the nodes of the cotyledonal node explants was significantly promoted by germinating cv Pag-asa 7 seeds and culturing the explants derived from these seedlings in BM (MS salts and B5 vitamins) with 1.0 mg l⁻¹ BA. Further addition of a non-ionic surfactant, Pluronic F-68 at 0.01 % or doubling of BA concentrations to 2.0 mg l⁻¹ during shoot initiation gave results comparable to those obtained using BM + 1.0 mg l⁻¹ BA alone. Therefore, the addition of 1.0 mg l⁻¹ BA alone to BM is sufficient for efficient induction of shoots from cotyledonal nodes of mungbean. Histological inspection of axillary shoot development in germinating seeds from day 1 to 4 revealed bigger and more advanced shoots in BA-preconditioned seedling over that of the control. This built-in advantage in BA-preconditioned explant sources was subsequently translated into improvement in shoot regeneration efficiency, an observation that justifies the use of BA-preconditioning to the optimize shoot regeneration response in many leguminous crop species. The regeneration system described herein was earlier proven to be widely applicable to all epigeal and the allotetraploid Vigna species comprising the subgenus Cерapotropis (Piper) Verdc. (Avenido and Hattori 1999, Avenido et al. 2001). Moreover, direct induction of multiple shoots from cotyledonary node explants being simple and efficient, provides a better tissue culture alternative for use in genetic transformation studies in Asiatic Vigna species.

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Literature Cited


Avenido and Hattori


