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Development of an Efficient Plant Regeneration System for the Selenium-hyperaccumulator *Astragalus racemosus* and the Nonaccumulator *Astragalus canadensis*

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Abstract. **A method of in vitro plant regeneration for both the selenium-hyperaccumulator *Astragalus racemosus* 'Cream Milkvetch' and the nonaccumulator *Astragalus canadensis* 'Canadian Milkvetch' was developed with two induction media, M1 and M2. The M1 and M2 contain Murashige and Skoog basal medium plus vitamins, 8.07 μM *N*-(2-chloro-4-pyridyl)-*N*′-phenylurea, 2.5% (w·v^{-1}) sucrose, 0.7% (w·v^{-1}) agar (pH 5.7), and 0.89 μM or 3.12 μM a-naphthaleneacetic acid, respectively. In vitro cultures were initiated on these two types of media with three types of explants: cotyledons, hypocotyls, and roots. More than 93% of cultured explants from both species could form calli or calli with shoots. With regard to shoot formation, *A. canadensis* could produce multiple shoots from all types of explants more efficiently than *A. racemosus*. The highest shoot induction was approximately three shoots per explant in *A. racemosus*, whereas *A. canadensis* could reach ≈10 shoots per explant. M1 could induce more shoots than M2 no matter what type of explant was used, but the overall induction rates were no significant difference. Among the three types of explants used, the cotyledons were the best explants for shoot induction in *A. canadensis*, whereas hypocotyls were the best in *A. racemosus*. In *A. racemosus*, shoots could also be obtained from calli on the rooting medium containing Murashige and Skoog basal plus vitamins, 2.84 μM indole-3 acetic acid, 2.5% (w·v^{-1}) sucrose, and 0.7% (w·v^{-1}) agar (pH 5.7). Approximately 43% of *A. canadensis* shoots and 19% of *A. racemosus* shoots could be rooted on the rooting medium.**

Selenium (Se) is an essential micronutrient for animals, including humans (Birringer et al., 2002), although for a long time, it was only known for its toxicity (Schwarz and Foltz, 1957). Recent studies have shown that Se-enriched plants are wanted for both cancer prevention (Finley, 2005) and Se phytoremediation (Berken et al., 2002; Sors et al., 2005). In nature, several Se-hyperaccumulating species of the genus *Astragalus* originating from seleniferous soils have been characterized (Trelease and Trelease, 1939). They cannot only tolerate high Se in soil, but can also hyperaccumulate Se at concentrations of up to 20 to 40 mg per gram of dry matter in their shoots when they grow under 2 to 10 ppm Se in natural soils (Davis, 1972). However, these Se accumulators are not edible plants for chemopreventive purposes and have very limited applications for Se phytoremediation because of their extremely slow growth and low biomass (Cunningham et al., 1997). In addition, the Se-hyperaccumulating plants cannot cross with any food plants as a result of genetic distance. Molecular cloning and genetic engineering may offer a better way to isolate genes related to the Se-hyperaccumulating property that could be transferred to other crop plants to create Se-enriched transgenic plants.

Currently, the functional analysis of the *Astragalus* genes related to Se accumulation is limited because there is no transformation and regeneration system available for the *Astragalus* species. So far, only one Se-accumulating gene, encoding selenocysteine methyltransferase, has been isolated from a Se hyperaccumulator, *Astragalus bisulcatus* (Neuhierl et al., 1999). Other genes that are also responsible for Se accumulation might exist. The discovery and study of additional Se-accumulating genes will help us to understand the mechanism of Se accumulation in Se hyperaccumulators and ultimately to develop transgenic plants with the Se-hyperaccumulating capacity. Gene transformation, however, needs reliable regeneration systems.

A previous report showed that calli could be induced from the hypocotyls of five Se-hyperaccumulator and three nonaccumulator species of *Astragalus* by including 13.57 μM 2,4-dichlorophenoxyacetic acid (2,4-D) in the induction medium (Ziebur and Shrift, 1971). Both the *A. racemosus* and *A. canadensis* species used in the present study were in their report. When those calli were subcultured, they failed to regenerate plants despite that incompletely developed roots and shoots were observed in some of their early subcultures, although they had been subcultured and maintained for several years (Ziebur and Shrift, 1971). Synthetic auxin, 2,4-D, is a strong hormone that is widely used to induce callus formation (Khanna and Raina, 1998). However, the concentration of 13.57 μM is considerably high for plant tissue culture, in which calli derived from an induction medium with such a high concentration of 2,4-D generally lose further dedifferentiation ability (Xie et al., 1995).

The objective of the current study was to establish a plant regeneration system for both the Se-hyperaccumulator *A. racemosus* and the nonaccumulator *A. canadensis*. To induce shoots, another more moderate auxin, a-naphthalene acetic acid (NAA), was chosen to combine with cytokinin 6-benzylaminopurine (BA) and cytokine-like *N*-(2-chloro-4-pyridyl)-*N*′-phenylurea (CPPU). CPPU has been proven to be able to effectively improve the efficiency of shoot formation and somatic embryogenesis in different plant species (Fiore et al., 2002; Millan-Mendoza, 1998; Murthy and Saxena, 1994; Nakajima et al., 2000; Tsuro et al., 1999; Zhang et al., 2005). We report our newly established plant regeneration system for *A. racemosus* and *A. canadensis*. Current results demonstrate that CPPU combined with NAA was good for *A. racemosus* and *A. canadensis* tissue cultures. Culture efficiencies of two species and three types of explants are compared.

Materials and Methods

Seedling preparation. Seeds of the Se-hyperaccumulator *A. racemosus* and the nonaccumulator *A. canadensis* were purchased from the Prairie Moon Nursery (Winona, MN). They were scarified by silica sand and cold-pretreated at 4 °C for 2 months before germination. Approximately 300 seeds of each species were germinated for experiments. Seeds were first surface-sterilized with 70% ethanol for 1 min and then rinsed with sterile water twice. They were then sterilized in a 40% Clorox (Clorox Professional Products Company, Oakland, CA; a commercial bleach containing 6% of the a.i., NaOCl) solution with 1% Tween 20 for 20 min and rinsed with sterile distilled water five times. Finally, sterilized seeds were germinated on Murashige and Skoog (MS) basal medium with vitamins (ID: M519; Phyto Technology Laboratories, Lenexa, KS)

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(Murashige and Skoog, 1962), 2.5% (w·v^{-1}) sucrose, and 0.7% (w·v^{-1}) agar (Acros Organics, Morris Plains, NJ). The pH was adjusted to 5.7 before autoclaving. Each 100 × 15-mm sterile petri dish (Fisher Scientific, Inc., Pittsburgh, PA) containing 20 mL of MS solid medium was placed with eight seeds. Petri dishes were then sealed with parafilm M (Fisher Scientific, Inc.) and incubated in a growth chamber under a condition of 16-h light and 8-h dark cycle at 25 °C with a light intensity of 50 μmol·m^{-2}·s^{-1}. After 4 to 7 d, most vital seeds started to germinate.

Explant preparation. In a preliminary test, all cotyledons were harvested from 7-d-old seedlings. In a formal experiment, cotyledons, hypocotyls, and roots of 3-week-old seedlings were separated for tissue culture. Roots and hypocotyls were cut into ≈0.6- to 0.8-cm long segments and cotyledons were cut into a 0.4- to 0.5-cm^2 area for culture. Because the cotyledons of these two species were small, almost all or the middle part of the cotyledon was needed for one piece of explant.

Preliminary culture test. Three pretesting experiments were conducted for callus and shoot inductions with the media containing an identical MS basal plus vitamins (Phyto Technology Laboratories), 2.5% (w·v^{-1}) sucrose, and 0.7% (w·v^{-1}) agar (pH 5.7) but different combinations of growth regulators. Five to 10 pieces of cotyledon explants were used to test each condition. Five explants were transferred onto a 100 × 15-mm sterile petri dish containing 20 mL of medium. The first pretesting experiment was a preliminary test with 8.07 μM CPPU and different concentrations of NAA. The induction medium containing 0.89 μM NAA and 8.07 μM CPPU was adapted from the medium used by Zhang et al. (2005) for Golden Pothos tissue culture. Three additional hormone combinations, 0, 3.12, and 5.35 μM NAA, were included with 8.07 μM CPPU. The second pretesting experiment was the test of 0, 4.52, or 13.57 μM of 2,4-D combined with 13.94 μM kinetin. In this pretest, we intended to repeat the method developed by Ziebur and Shrift (1971) using a lower concentration of 2,4-D. The third pretesting experiment was the test of 4.49 μM BA combined with 0, 0.89, 3.12, or 5.35 μM NAA.

Induction media, explants, and experimental design in the formal experiment. Based on the pretesting results, two best induction media with 0.89 μM NAA + 8.07 μM CPPU (named M1) and 3.12 μM NAA + 8.07 μM CPPU (named M2) were selected to evaluate tissue culture efficiencies for *A. racemosus* and *A. canadensis* with three different types of explants: cotyledons, hypocotyls, and roots. Every eight explants were cultured onto a 100 × 15-mm sterile petri dish containing 20 mL of medium. Each petri dish was counted as a replicate. A total of nine petri dishes (representing nine replicates) were used for each treatment. All petri dishes were sealed with parafilm M and incubated in a growth chamber under a 16-h light and 8-h dark cycle at 25 °C with a light intensity of 50 μmol·m^{-2}·s^{-1}. After 5 weeks of initial culture, all explants with induced calli, shoots, or both were transferred onto fresh medium for another 3 weeks to induce more shoots and to maintain the growth of shoots.

Data collection and analysis. Numbers of calli and shoots from each plate were counted after 8 weeks of culture. An average of 72 explants from a total of nine petri dishes for each treatment was taken for statistical analysis. Data for the percentage of explants with calli and shoots, or callus only, and the number of shoots produced per explants were subjected to statistical analysis. The variances among the 12 treatments were analyzed by the JMP software (SAS Institute, Cary, NC), and their means separation was performed using least significant means difference Student's *t* test at $P \leq 0.05$.

Rooting. After recording callusing and shooting rates, a total of 108 shoots from *A. canadensis* cotyledon culture and 94 shoots from *A. racemosus* cotyledon culture with more than two small true leaves and a length greater than 1 cm were isolated individually for rooting. The rooting medium was MS basal plus vitamins, 2.84 μM indole-3 acetic acid (IAA), 2.5% (w·v^{-1}) sucrose, and 0.7% (w·v^{-1}) agar (pH 5.7). Five to six shoots were transplanted in a 100 × 20-mm sterile petri dish (Fisher Scientific, Inc.) containing 25 mL of rooting medium. All petri dishes were sealed with parafilm M and incubated in a growth chamber under a 16-h light and 8-h dark cycle at 25 °C with a light intensity of 50 μmol·m^{-2}·s^{-1}. Every 4 weeks, shoots were transferred to a fresh rooting medium for a total of an 8-week period. Some calli derived from *A. racemosus* cotyledon cultures were also transferred to rooting medium for shoot induction and rooting test.

Results

Preliminary culture test. To establish a plant regeneration system for the Se-hyperaccumulator *A. racemosus* and the nonaccumulator *A. canadensis*, we first pretested the induction media with 11 different growth regulator combinations using five to 10 pieces of cotyledon explants per condition. Preliminary testing results showed that both induction media with 0.89 μM NAA + 8.07 μM CPPU (named M1) and 3.12 μM NAA + 8.07 μM CPPU (named M2) were good for shoot regeneration (Table 1). The M1 was adapted from the medium used by Zhang et al. (2005) for Golden Pothos tissue culture that was shown to be very efficient in terms of the percentages of explants with calli and shoots and the numbers of induced shoots per explant. After culturing for 8 weeks, all cotyledon explants from *A. canadensis* and nearly half of cotyledon explants from *A. racemosus* produced calli with shoots on these two media. The M2 seemed to perform even better than the M1. Remaining growth regulator combinations either had less ability to induce calli with shoots or could only produce calli. Based on this preliminary test, both M1 and M2 media were selected to develop plant regeneration systems and study their culture responses for *A. racemosus* and *A. canadensis*.

Callus and shoot induction with A. canadensis *and* A. racemosus. Although the cotyledons are considered as the best explants for tissue culture in most species (Murthy et al., 1996), different tissue explants may have various culture responses in different species (Komai et al., 1996). To establish an efficient culture system, three different types of explants from each species: cotyledons, hypocotyls, and roots were tested with the induction media M1 and M2. After culturing for 4 to 6 d, our first observation was that most explant tissues from *A. racemosus* and *A. canadensis* were expanded on both M1 and M2 media. Calli appeared on cotyledon explants in the initial 2-week culture in both species (Fig. 1A–B). Some green shoots together with calli were observed in *A. canadensis* cotyledon explants in the third week, and more green shoots emerged after an additional 2 to 3 weeks of culture (Fig. 1A, E), whereas shoots were first observed after 5 to 6 weeks in *A. racemosus* cotyledon culture (Fig. 1F). These results

Table 1. The number of cotyledon explants from *A. canadensis* and *A. racemosus* forming calli or calli with shoots on different pretesting media.[z]

Species	*A. canadensis*			*A. racemosus*		
Growth regulators[y]	Total explants tested	Calli with shoots	Calli only	Total explants tested	Calli with shoots	Calli only
8.07 μM CPPU	5	2	1	5	1	2
8.07 μM CPPU + 0.89 μM NAA	5	4	0	5	2	3
8.07 μM CPPU + 3.12 μM NAA	5	5	0	5	3	2
8.07 μM CPPU + 5.35 μM NAA	5	3	1	5	1	2
13.94 μM kinetin	10	0	0	10	0	0
13.94 μM kinetin + 4.52 μM 2,4-D	10	0	5	10	0	5
13.94 μM kinetin + 13.57 μM 2,4-D	10	0	6	10	0	5
4.49 μM BA	5	1	1	5	0	1
4.49 μM BA + 0.89 μM NAA	5	1	1	5	0	1
4.49 μM BA + 3.12 μM NAA	5	2	1	5	1	0
4.49 μM BA + 5.35 μM NAA	5	1	2	5	1	1

[z]Each petri dish contained five cotyledon explants. Each treatment has either one or two petri dishes.
[y]The basic medium contained Murashige and Skoog mineral salts plus vitamins, 2.5% (w·v^{-1}) sucrose, and 0.7% (w·v^{-1}) agar (pH 5.7). The growth condition was under a 16-h light and 8-h dark cycle at 25 °C with a light intensity of 50 μmol·m^{-2}·s^{-1}.
CPPU = cytokine-like *N*-(2-chloro-4-pyridyl)-*N*′-phenylurea; NAA = a-naphthalene acetic acid; 2,4-D = 2,4-dichlorophenoxyacetic acid; BA = 6-benzylaminopurine.

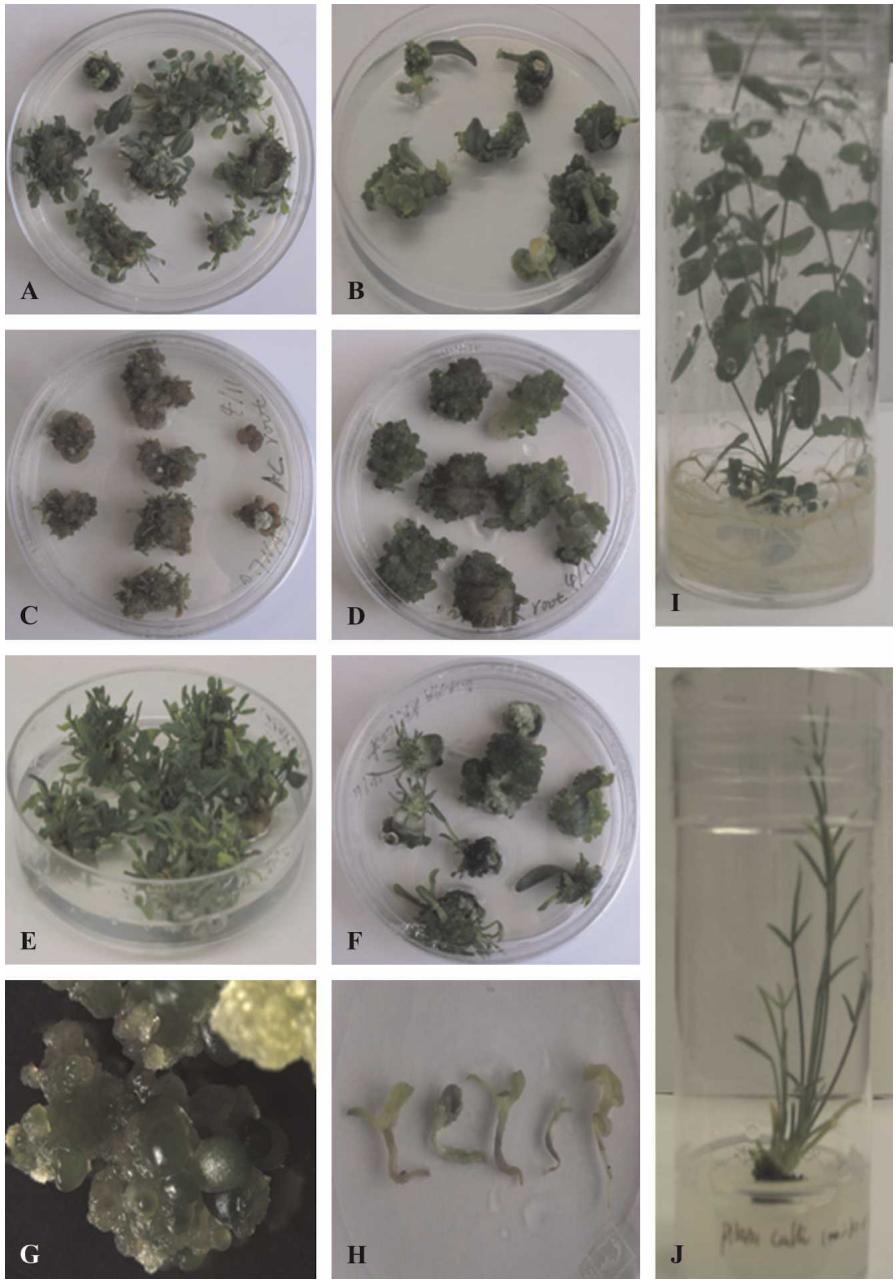

Fig. 1. Plant regeneration of both the selenium-hyperaccumulator *A. racemosus* and the nonaccumulator *A. canadensis*. (**A**) *A. canadensis* cotyledon culture with shoots and calli; (**B**) *A. racemosus* cotyledon culture with calli and a few shoots; (**C**) *A. canadensis* root culture with shoots and calli; (**D**) *A. racemosus* root culture with calli; (**E**) multiple shoots in *A. canadensis* cotyledon culture; (**F**) multiple shoots in *A. racemosus* cotyledon culture; (**G**) calli derived from *A. racemosus* cotyledon culture; (**H**) small plantlets from *A. racemosus* callus subculture; (**I**) plantlet of *A. canadensis*; (**J**) plantlet of *A. racemosus*.

imply that shoots were induced more quickly and easily from *A. canadensis* than *A. racemosus* cotyledons. Meanwhile, both species of hypocotyl and root explants expanded and formed calli during the first 3 to 4 weeks of culture, and a few small shoots appeared several weeks later (Fig. 1C–D). Calli derived from all three types of *A. racemosus* explants and *A. canadensis* cotyledons were green (Fig. 1A, B, D), whereas some calli from *A. canadensis* hypocotyls and roots appeared to be rust-colored (Fig. 1C). However, the regenerated shoots derived from rust-colored calli were normal and green (Fig. 1C). Most calli were firm and could produce shoots, whereas a small number of calli was friable to soft and died easily in the later culture in both species.

Callus and shoot induction rates. The culture results showed that both induction media were good for *A. canadensis* and *A. racemosus* tissue culture, and 93% to 100% of placed explants from both species and the three types of explants could produce either calli or calli with shoots after 8 weeks of culture (Table 2). A higher percentage of explants having calli with shoots was obtained in M1 medium than in M2 medium in all treatments. For different species, ≈68.1% to 100% of explants from *A. canadensis* could produce calli with shoots, whereas those of *A. racemosus* were less capable with only ≈12.5% to 70.8% producing calli with shoots. However, the remaining 27.8% to 83.3% explants of *A. racemosus* could produce calli without shoots.

Compared with the different explants, the percentage of the calli formed with shoots was similar among the three types of explants in *A. canadensis*. They all had a high callus-with-shoot induction rate (90.3% to 100%) except the root explants in M2 medium, in which the rate was 68.1%. In *A. racemosus*, explants from hypocotyls had the best ability to produce calli with shoots (70.8% in M1 and 54.2% in M2) followed by cotyledons (45.8% in M1 and 37.5% in M2) and roots (23.6% in M1 and 12.5% in M2).

The number of induced shoots. The induced shoots were compared after 8 weeks of culture. *A. canadensis* had a greater ability to produce shoots than *A. racemosus* in all three types of explants on the two induction media (Table 3). Each explant from *A. canadensis* cotyledon could produce 10 shoots, whereas explants from *A. racemosus* produced fewer than three shoots. These results further suggest that *A. canadensis* is better equipped than *A. racemosus* to be able to produce shoots under the current culture conditions. For shoot induction, M1 induced more shoots than M2 no matter what type of explant was used, but the overall induction rates were not very different. Moreover, the three types of explants had different abilities to produce shoots. In *A. canadensis*, cotyledons were the best explants for shoot induction with nine to 10 shoots per explant, whereas hypocotyls and roots were equally low in shoot induction. However, for *A. racemosus*, hypocotyls were the best explants for shoot induction with approximately two to three shoots per explant followed by cotyledons and roots.

Shoots from A. racemosus *calli.* Although many calli of *A. racemosus* did not have any shoot emerge (Fig. 1G), we still transferred some of them onto rooting medium for shoot induction and rooting tests. Surprisingly, after subculture on rooting medium for ≈8 weeks, some calli began to have shoots with leaves and roots as well (Fig. 1H). This result indicated that some *A. racemosus* calli could be used to induce shoots in rooting medium.

Rooting. To examine rooting efficiency, individual shoots longer than 1 cm were isolated from the cluster of shoots and were put on a rooting medium that consisted of MS basal plus 2.84 μM IAA. We isolated 108 shoots from *A. canadensis* cotyledon culture and 94 shoots from *A. racemosus* cotyledon culture for the rooting test. The results showed that only a few shoots could produce roots during the initial 4 weeks of culture on the rooting medium. After they were subcultured on new rooting medium for another 4 weeks, 49 shoots of *A. canadensis* and 18 shoots of *A. racemosus* could be

Table 2. The percentage of explants from *A. canadensis* and *A. racemosus* forming calli or calli with shoots on two induction media.[z]

Species	Medium[y]	Explants	Calli with shoots (%)	Calli only (%)	Total culture response (%)
A. canadensis	M1	Cotyledons	98.6 ± 4.2 a[x]	1.4 ± 4.2 f	100
	M1	Hypocotyls	98.6 ± 4.2 a	0	98.6
	M1	Roots	100 ± 0 a	0	100
	M2	Cotyledons	95.8 ± 8.8 a	4.2 ± 8.8 f	100
	M2	Hypocotyls	90.3 ± 13.7 a	9.7 ± 13.7 f	100
	M2	Roots	68.1 ± 14.1 b	30.6 ± 14.1 de	100
A. racemosus	M1	Cotyledons	45.8 ± 33.7 c	47.2 ± 26.4 cd	93.0
	M1	Hypocotyls	70.8 ± 25.8 b	27.8 ± 24.0 e	98.6
	M1	Roots	23.6 ± 22.9 de	76.4 ± 22.9 ab	100
	M2	Cotyledons	37.5 ± 25.8 cd	61.1 ± 25.3 bc	98.6
	M2	Hypocotyls	54.2 ± 30.0 bc	45.8 ± 30.0 cd	100
	M2	Roots	12.5 ± 19.8 e	83.3 ± 25 a	95.8

[z]Data represent the averages of nine petri dishes per treatment. Each petri dish contained eight explants.
[y]The M1 medium contained Murashige and Skoog (MS) mineral salts plus vitamins, 0.89 μM NAA, 8.07 μM CPPU, 2.5% (w·v^{-1}) sucrose, and 0.7% (w·v^{-1}) agar (pH 5.7). M2 medium contained MS mineral salts plus vitamins, 3.12 μM of NAA with 8.07 μM CPPU, 2.5% (w·v^{-1}) sucrose, and 0.7% (w·v^{-1}) agar (pH 5.7). Cultured under a condition of 16-h light and 8-h dark cycle at 25 °C with a light intensity of 50 μmol·m^{-2}·s^{-1}.
[x]Mean separation within columns was by least significant means difference Student's *t* test ($P \leq 0.05$).

Table 3. The number of induced shoots per explant from *A. canadensis* and *A. racemosus* with three types of explants on two induction media.[z]

Species	Medium[y]	Explants	Shoots per explant
A. canadensis	M1	Cotyledons	10.0 ± 1.3 a[x]
	M1	Hypocotyls	4.9 ± 0.5 b
	M1	Roots	4.9 ± 0.8 b
	M2	Cotyledons	9.0 ± 2.9 a
	M2	Hypocotyls	4.1 ± 1.8 bc
	M2	Roots	1.9 ± 0.8 def
A. racemosus	M1	Cotyledons	1.6 ± 1.8 def
	M1	Hypocotyls	3.0 ± 1.9 cd
	M1	Roots	0.7 ± 0.8 f
	M2	Cotyledons	1.0 ± 0.8 ef
	M2	Hypocotyls	2.3 ± 1.9 de
	M2	Roots	0.6 ± 0.7 f

[z]Data represent the averages of nine petri dishes per treatment. Each petri dish contained eight explants.
[y]The M1 medium contained Murashige and Skoog (MS) mineral salts plus vitamins, 0.89 μM NAA, 8.07 μM CPPU, 2.5% (w·v^{-1}) sucrose, and 0.7% (w·v^{-1}) agar (pH 5.7). M2 medium contained MS mineral salts plus vitamins, 3.12 μM of NAA with 8.07 μM CPPU, 2.5% (w·v^{-1}) sucrose, and 0.7% (w·v^{-1}) agar (pH 5.7). Cultured under a condition of 16-h light and 8-h dark cycle at 25 °C with a light intensity of 50 μmol·m^{-2}·s^{-1}.
[x]Mean separation within columns was by least significant means difference Student's *t* test ($P \leq 0.05$).

rooted. Rooting rates of *A. canadensis* and *A. racemosus* shoots were 43% and 19%, respectively.

Discussion

Our culture results demonstrate that the plant regeneration system was successfully established for both the Se-hyperaccumulator *A. racemosus* and the nonaccumulator *A. canadensis* with M1 and M2 media. *A. canadensis* is better than *A. racemosus* for shoot induction under the current conditions. However, because the calli derived from *A. racemosus* tissue culture could develop into shoots on rooting medium, the system we developed is sufficient for plant regeneration from both species.

Different genotypes having different culture responses under the same induction conditions have been documented before, which may result from different endogenous hormone levels in different genotypes (Kasha et al., 1990). This is also true for different tissues used for shoot induction. Among all of the different types of explants used in our study, culture responses were different in term of the number of shoots per explant. The explants from cotyledons were the best in *A. canadensis* culture, whereas those from hypocotyls were the best for shoot induction in *A. racemosus*. Different tissue explants having different culture responses may result from an uneven distribution of endogenous hormones in different parts of a plant (Okubo et al., 1991).

Besides the genotypes and tissue explants that affect shoot induction rates, another major factor determining whether shoots can or cannot be induced is the induction medium, especially its growth regulator combination. In our study, NAA combined with a cytokine-like CPPU, a synthetic phenylurea derivative (Takahashi et al., 1978), proved to be effective for *A. racemosus* and *A. canadensis* tissue culture, leading to the production of either calli or calli with shoots in most of the placed explants. Other growth regulator combinations tested in our preliminary work indicated that CPPU was better than BA and that NAA was better than 2,4-D for our culture purposes. These preferences toward certain types of growth regulators might well be the result of species specificity. The better effect of CPPU on the callus/shoot induction may result from its strong cytokinin function. Compared with BA, CPPU is a strong cytokinin for cell division and callus proliferation (Takahashi et al., 1978). The other reason is the CPPU is more stable and has a different metabolism compared with the natural cytokine, BA (Mok and Mok, 2001). The effect of CPPU on increasing cell division and proliferation is through upregulating the expression of the *CycD3* gene, which encodes the CycD cyclins involving regulating the G1 phase during cell cycle (Li et al., 2003).

Calli derived from all three types of *A. racemosus* explants and *A. canadensis* cotyledons were green, whereas some calli from *A. canadensis* hypocotyls and roots appeared to be rust-colored, which was somehow different from the report of Ziebur and Shrift (1971). In their hypocotyl culture, calli from *A. canadensis* were green, whereas those from *A. racemosus* were rust-colored, beige, or pale green. This difference may be caused by the different induction medium used, although the actual cause of the rust-colored calli is not clear. The exact cause of rust-colored calli in *A. canadensis* needs to be further investigated. Interestingly, this rust color was also observed in our cell suspension culture. Both cell suspension lines derived from *A. canadensis* cotyledon and root calli showed a pale rust color and grew relatively faster than those from *A. racemosus*, which were white to yellow (our unpublished data).

In conclusion, this is the first report of plant regeneration from both the Se-hyperaccumulator *A. racemosus* and the nonaccumulator *A. canadensis* in three different types of explants. Although the Se-hyperaccumulating characteristic has been recognized for many decades in some *Astragalus* species, only one gene, encoding selenocysteine methyltransferase, has been isolated and confirmed to be related to the Se accumulation in *A. bisulcatus* leaves. These established plant regeneration and callus induction systems for both *A. racemosus* and *A. canadensis* will allow us to develop a transformation system and cell suspension culture lines for study of Se accumulation. The cell suspension culture lines could also be used for Se treatment to induce Se-related differentially expressed genes, and a future *Astragalus* transformation system will facilitate the isolation and characterization of more Se-accumulating genes.

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