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ORIGINAL PAPER

Adventitious shoot regeneration from cotyledonary explants of rapid-cycling fast plants of *Brassica rapa* L

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Abstract Rapid-cycling fast plants (*Brassica rapa*; RCBr) is also known as Wisconsin Fast Plant and is widely used in K-12 and undergraduate studies. RCBr has a short generation time (seed-to-seed in 30–60 days), which allows for the completion of experiments in a semester. Previous studies have shown that cotyledonary explants with attached petioles are capable of generating shoots. However, there is no published adventitious shoot regeneration protocol to date. Sterile cotyledonary explants were excised; all edges and petioles were removed. Five-day-old cotyledonary explants produced shoots on a Murashige and Skoog medium containing 1.5 mg/L thiadiazuron (TDZ) and 0.5 mg/L 1-naphthaleneacetic acid (NAA) (FPM I) at a mean rate of 8.8%. This rate increased to 14.8% in explants placed on FPM I medium supplemented with 5.0 mg/L silver nitrate ($AgNO_3$) (SRM 2). The rate increased to 32.5% when 5-day-old explants, excised from the part of the cotyledon nearest to the petiole, were placed adaxial side up on SRM 2 medium. The shoot regeneration rate increased to 44.5% using 4-day-old cotyledonary explants. A shoot regeneration rate of 23% was observed among 9-day-old leaf explants. Shoots from cotyledonary explants were elongated on basal medium with 0.5 mg/L NAA, rooted on basal medium, and later acclimatized. This is the first report of shoot regeneration from cotyledonary explants of rapid-cycling *Brassica rapa* without pre-existing meristematic tissues.

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Introduction

The Brassicaceae family of plants has over 350 genera and 3,000 species. Several of the species within the genus *Brassica* are economically important crop plants such as turnip (*B. rapa*), canola (*B. napus*), and cabbage and broccoli (*B. oleracea*) (Musgrave 2000). Rapid-cycling fast plants, also known as Wisconsin Fast Plant (*Brassica rapa*; RCBr), are genetically altered *Brassica* populations (Williams and Hill 1986). They have a short life cycle (seed to seed in less than 60 days), high female sterility, rapid seed maturation, and an absence of seed dormancy (Teo et al. 1997). They are used extensively in K-12 and undergraduate education. Educational topics that are addressed using RCBr include plant growth and development (seed germination, growth responses, photosynthesis and nutrition); reproductive biology (flower morphology and pollination); and genetics (mendelian inheritance using mutants, selection) (Musgrave 2000). There are several websites that have resources for educators interested in RCBr as well as several companies that sell educational kits.

RCBr is diploid, having 20 chromosomes and a genome that is only four fold larger than *Arabidopsis thaliana* (the most frequently used plant model and a member of the same family) (Cardoza and Stewart 2004). In contrast to *A. thaliana*, which is self-fertilized, RCBr has naturally occurring self-incompatibility genes which significantly reduce the pollination of stigmas by their own pollen (Musgrave 2000).

One limitation of RCBr as a plant model system is the scarcity of published reports on tissue culture; whilst another limitation is the lack of a reproducible transformation

protocol (Millam et al. 1991; Takasaki et al. 1996). Teo et al. (1997) achieved a shoot regeneration rate of 20% using 3-day-old RCBr cotyledonary explants regenerated on medium containing Murashige and Skoog (1962) basal salts and vitamins with 20 μM (4.5 mg/L) 6-benzylaminopurine (BA) and 2 μM (0.4 mg/L) 1-naphthaleneacetic acid (NAA). This regeneration rate increased to 33% when the medium was supplemented with 2 μM aminoethoxyvinylglycine (0.4 mg/L; AVG). However, shoots arose only from cotyledonary ends that had attached petioles (Teo et al. 1997). A regeneration rate of 97% was obtained from 3-day-old cotyledons of rapid-cycling *B. oleracea* on medium containing MS basal salts and vitamins and 5.0 μM TDZ, however, the cotyledons had attached petioles (Cheng et al. 2001).

Cotyledonary explants with attached petioles have preformed meristematic tissues, which are already programmed to differentiate into organs (organogenesis). The major problem with this type of regeneration is that transferring genes into these tissues is problematic due to the increased possibility of chimeras. This usually results in a longer selection time for fully transformed plants (Geier and Sangwan 1996). In addition, there is no reported transformation protocol using this type of RCBr explants or for any other type of explants.

The current aim of this research is to develop an adventitious shoot regeneration protocol from cotyledonary explants of rapid-cycling *Brassica rapa*. We then intend to use this method to produce transgenic plants via *Agrobacterium tumefaciens* and biolistics. These new protocols will complement the experiments available to K-12 and undergraduate students in plant molecular biology.

Materials and methods

Plant material and in vitro germination

All chemicals used were from Caisson Laboratories (North Logan, UT) unless otherwise stated. Seeds of rapid-cycling fast plants (*B. rapa:* RCBr) (Carolina Biological Co., Burlington, NC) were surface sterilized in 10% bleach solution, containing 0.1% SDS, and stirred gently for 20 min. The seeds were washed three times with sterile de-ionized water and placed on fast plant growth medium (FPM) in magenta boxes. FPM consisted of MS (Murashige and Skoog 1962) basal salts and vitamins, 30 g/L sucrose, and 8 g/L phytagar. The pH was adjusted to 5.8 and the medium was autoclaved for 35 min at 121 psi. All media used for shoot regeneration experiments were derived from FPM. All experiments were conducted under 16 h photoperiod using cool white fluorescent lights (140 μmol m^{-2} s^{-1}).

Shoot regeneration from RCBr cotyledonary and leaf explants

Cotyledons were excised from 5-day-old seedlings and all edges were removed. Care was taken to ensure that the petioles were removed. Cotyledonary explants were placed on 15 different media, containing varying concentrations of thiadiazuron (TDZ) and 1-naphthaleneacetic acid (NAA). Explants were observed every 2 weeks for 6 weeks for callus/meristemoid production and shoot regeneration.

Five-day-old cotyledonary explants were placed on FPM I (FPM supplemented with 1.5 mg/L TDZ and 0.5 mg/L NAA) containing four different concentrations of silver nitrate ($AgNO_3$). Explants were observed every 2 weeks for 6 weeks for callus/meristemoid production and shoot regeneration.

Five-day-old cotyledonary explants were excised from the proximal (closest to the petiole) or the distal end of the cotyledon and placed abaxial (upper epidermis touching the medium) or adaxial side up (lower epidermis touching the medium) on SRM 2 (shoot regeneration medium: FPM supplemented with 1.5 mg/L TDZ, 0.5 mg/L NAA and 5.0 mg/L $AgNO_3$). Explants were observed every 2 weeks for 6 weeks for callus/meristemoid production and shoot regeneration.

Cotyledonary explants were cut on the same day from 6, 5, 4, and 3-day-old plants (all were cut from the proximal end except 3-day-old) and placed adaxial side up on SRM 2. These explants were observed every 2 weeks for 6 weeks for callus/meristemoids production and shoot regeneration.

Explants were excised from the proximal end of 9-day-old RCBr leaves and placed adaxial side up on SRM 2. Explants were observed every 2 weeks for 6 weeks for callus/meristemoid production and shoot regeneration.

Elongation, rooting, and acclimatization of regenerated shoots

Three-week-old shoot clumps were excised and placed on FPM containing 0.5 mg/L NAA (shoot elongation medium: SEM). Shoots were observed every 2 weeks for 6–8 weeks.

Elongated shoots were excised from shoot clumps on SEM and transferred to FPM (hormone free medium) for rooting. Plants were observed every week for 6 weeks for root formation. After 3 weeks, rooted shoots were transferred from FPM to sterile soil and acclimatized.

Statistical analyses

All shoot regeneration experiments consisted of ten explants per plate with five replications per treatment and were repeated at least once. Factorial treatments in a completely

randomized design were used in experiments using TDZ, NAA and $AgNO_3$. All experiments were analyzed using PROC GLM of the SAS statistical package (Cary, NC). Means were separated using Fisher's Protected LSD using a significance value of 0.05.

Results

Effects of TDZ, NAA, and $AgNO_3$ concentrations

No shoot regeneration from 3, 4, 5, or 6-day-old cotyledonary explants (no attached petioles) of RCBr was observed on media containing varying concentrations of 6-benzylaminopurine (BA) and NAA (data not shown). Additionally, no shoot regeneration was observed on media containing TDZ alone (Table 1; FPM A to E). There were no significant differences in percentage of explants producing shoots with increasing TDZ and NAA concentrations (Table 1; FPM F to J; FPM K to O). Higher concentrations of TDZ and NAA however, increased the number of adventitious shoots. On average, those explants on FPM K–O produced more shoots per responding explant (between 4.5 and 13.2) than those on FPM F-J (between 2.7 and 16.0) after 6 weeks (Table 1). If the petioles were not excised properly, shoots elongated directly (non-adventitious) from the nodal tissues at a faster rate (within 2 weeks), rather than meristemoids, and similar regeneration rates to Teo et al. (1997) were obtained.

FPM I was chosen as the best medium for further studies because it had a relatively high mean regeneration rate (8.8%) and produced one of the highest mean number of shoots per responding explant (13.7: Table 1). These explants produced very little callus (Fig. 1a) and meristemoids were observed within 2 weeks (Fig. 1b). Although FPM K and O had higher mean shoot regeneration rates (12.5 and 9.5%, respectively), neither were significantly different from FPM I. They also produced fasciated shoots (more difficult to elongate) and more shoot clumps after 6 weeks.

To increase the shoot regeneration rate, experiments were conducted with varying concentrations of silver nitrate. SRM 2 medium (1.5 mg/L TDZ, 0.5 mg/L NAA and 5.0 mg/L $AgNO_3$) produced the highest mean regeneration rate (14.8%) and had the largest shoot production (20.2 shoots/responding explant) after 6 weeks (Table 2; Fig. 1c, d).

Effects of orientation and position

The effect of explant orientation (adaxial vs. abaxial) and position (basal vs. distal end of the cotyledon) on shoot regeneration were investigated. Cotyledonary explants placed ADP (adaxial side up from the proximal end) on SRM 2 yielded the highest mean shoot regeneration rate (32.5%) and produced the greatest mean number of shoots per responding explant (13.7: Table 3). Explants placed ADD (adaxial side up from the distal end) and ABP (abaxial side

Table 1 Effects of TDZ and NAA on shoot regeneration from cotyledonary explants of rapid-cycling *B. rapa*

Medium	TDZ (mg/L)	NAA (mg/L)	Mean percentage (%) of explants producing shoots (mg/L)	Mean number of shoots per responding explants
FPM A	0.3	0.0	0.0 b	0.0 c
FPM B	0.6	0.0	0.0 b	0.0 c
FPM C	0.9	0.0	0.0 b	0.0 c
FPM D	1.5	0.0	0.0 b	0.0 c
FPM E	3.0	0.0	0.0 b	0.0 c
FPM F	0.3	0.5	3.0 ± 4.2 ab	2.7 ± 3.7 bc
FPM G	0.6	0.5	0.0 b	0.0 c
FPM H	0.9	0.5	1.0 ± 1.4 b	9.5 ± 13.4 abc
FPM I	1.5	0.5	8.8 ± 1.8 ab	13.7 ± 5.2 ab
FPM J	3.0	0.5	6.3 ± 5.3 ab	16.7 ± 4.7 a
FPM K	0.3	1.0	12.5 ± 14.8 a	12.5 ± 0.7 ab
FPM L	0.6	1.0	5.8 ± 2.5 ab	9.7 ± 8.0 abc
FPM M	0.9	1.0	1.3 ± 1.8 b	4.5 ± 6.4 bc
FPM N	1.5	1.0	4.5 ± 0.7 ab	11.8 ± 7.4 abc
FPM O	3.0	1.0	9.5 ± 7.8 ab	13.2 ± 7.6 ab

Five-day-old cotyledonary explants were excised and placed on FPM containing various concentrations of TDZ and NAA. They were observed every 2 weeks for 6 weeks for shoot production. Means with the same letter are not statistically significant at $P \leq 0.05$

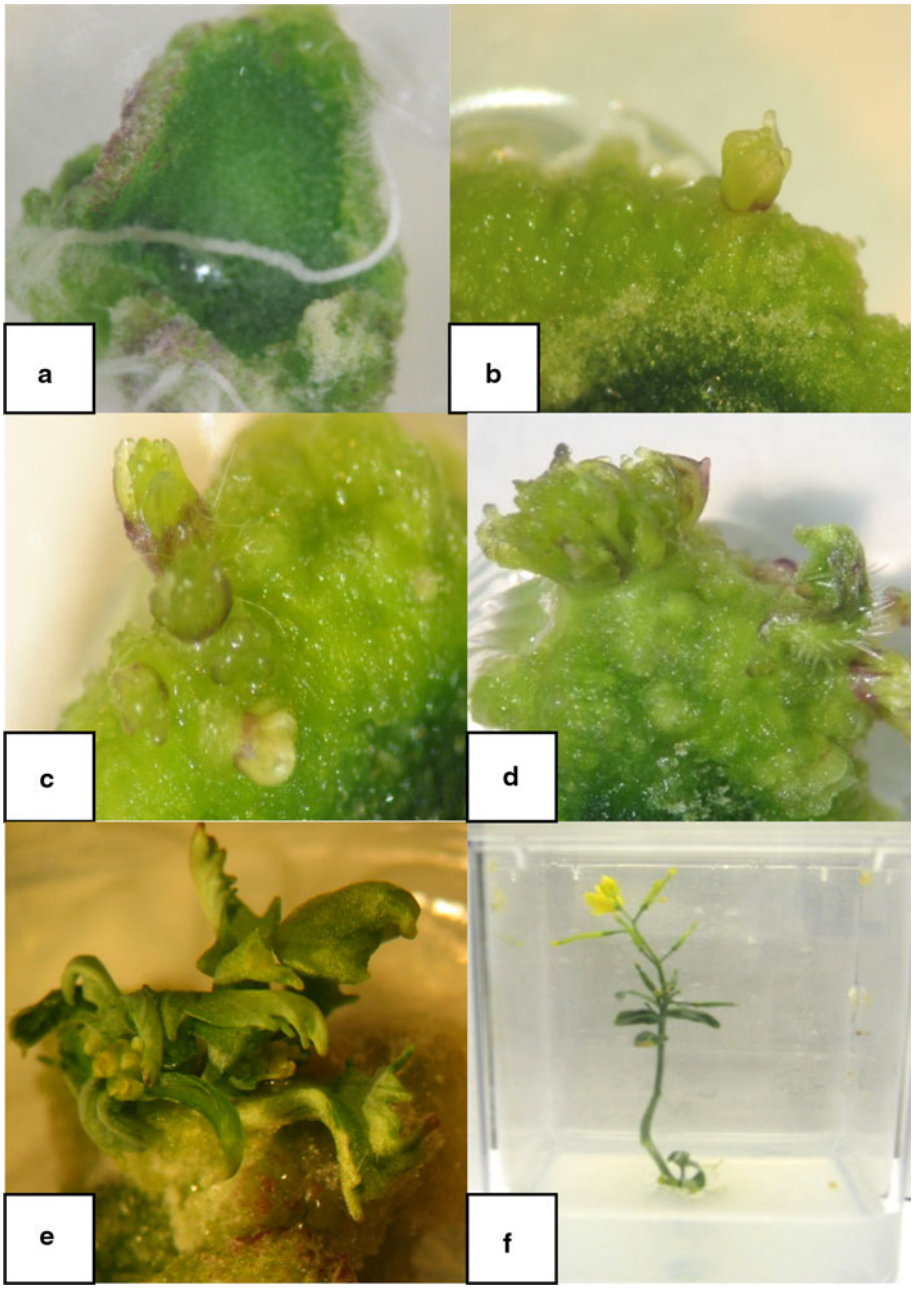

Fig. 1 Regeneration of plants from cotyledonary explants of rapid-cycling *Brassica rapa*: **a** explants producing callus and some roots after 1 week on shoot regeneration medium; **b** explant producing meristemoids after 2 weeks; **c** meristemoids elongating after 3 weeks on shoot regeneration medium; **d** clumps of shoots after 5 weeks on shoot regeneration medium; **e** elongating shoots after 2 weeks on elongation medium; **f** rooted explants after 2 weeks on rooting medium

Table 2 Effects of silver nitrate ($AgNO_3$) on shoot regeneration from cotyledonary explants of rapid-cycling *B. rapa*

Medium	$AgNO_3$ (mg/L)	Mean percentage (%) of explants producing shoots	Mean number of shoots per responding explant
SRM 1	0.0	5.2 ± 3.2 a	6.5 ± 3.5 a
SRM 2	5.0	14.8 ± 3.9 a	20.2 ± 5.2 a
SRM 3	10.0	14.7 ± 1.9 a	8.5 ± 4.2 a
SRM 4	20.0	4.5 ± 6.3 a	6.2 ± 8.7 a

Five-day-old cotyledonary explants were excised and placed on SRM containing 1.5 mg/L TDZ, 0.5 mg/L NAA and various concentrations of $AgNO_3$. They were observed every 2 weeks for 6 weeks for shoot production. Means with the same letter are not statistically significant at $P \leq 0.05$

Table 3 Position and orientation effects on shoot regeneration from cotyledonary explants of rapid-cycling *B. rapa*

Orientation/position	Mean percentage (%) of explants producing shoots	Mean number of shoots per responding explant
Adaxial/Proximal (ADP)	32.5 ± 7.1 a	13.7 ± 1.1 a
Adaxial/Distal (ADD)	14.0 ± 5.7 ab	14.4 ± 1.7 a
Abaxial/Proximal (ABP)	19.0 ± 12.7 ab	11.2 ± 0.8 a
Abaxial/Distal (ABD)	6.1 ± 5.6 b	16.8 ± 8.8 a

Five-day-old cotyledonary explants were cut in a combination of orientations (abaxial vs. adaxial) and positions (proximal vs. distal) and placed on SRM 2 containing 1.5 mg/L TDZ, 0.5 mg/L NAA, and 5.0 mg/L $AgNO_3$. Explants were observed every 2 weeks for 6 weeks for shoot production. Means with the same letter are not statistically significant at $P \leq 0.05$

up from the proximal end) produced fewer shoots (14.5 and 19%, respectively) than ADP. Explants placed ABD (abaxial side up from the distal end) had the lowest shoot regeneration rate (6.1%) after 6 weeks (Table 3).

Effects of age of explant

Three-day-old explants had the highest shoot regeneration rate at 47.5% and produced the highest mean number of shoots per responding explant (10.3) when the proximal end of explants were placed adaxial side up on SRM 2 (Table 4). However, these explants were very small and thus, too difficult to cut. By the fourth day, the cotyledonary explants were larger, easier to handle, and excision based on orientation and position could be maintained.

Four-day-old explants produced a mean shoot regeneration rate of 44.5% with each explant averaging 10.2 shoots after 6 weeks (Table 4). This data was not significantly different from that of 3-day-old explants. The older explants (5 and 6-day-old) produced significantly less shoots (18 and 20%, respectively) when compared with 3 and 4-day-old explants (Table 4).

A shoot regeneration rate of 23% and a mean of 6.1 shoots per responding explant were observed in 9-day-old leaf explants of RCBr. These explants were more fragile than the cotyledonary explants, as they wilted faster once they were excised from the plants. However, they grew larger and produced more meristemoids; but shoot clumping and elongation were similar to cotyledonary explants.

Elongation, rooting, and acclimatization of regenerated shoots

To increase shoot elongation, 21 clumps of shoots (about 15 shoots/clump: Fig. 1e) from cotyledonary explants were transferred from SRM 2 to SEM (FPM containing 0.5 mg/L NAA). Thirty-nine shoots elongated within 2 weeks. In total, 71 shoots elongated in 6 weeks. The 39 shoots were transferred to FPM for rooting, where 11 of the 39 shoots (28.2%) developed roots and flowers in vitro within 3 weeks [Fig. 1f]. After 6 weeks, the number of rooted plants rose to 61%. Eight of the 11 plants (72%) that produced roots within 3 weeks, were successfully acclimatized. A total of 21 plants (88%) were acclimatized, and grew to maturity with fertile flowers and seeds.

Discussion

Rapid-cycling *Brassica rapa* is one of the most recalcitrant species for shoot organogenesis. Most tissue culture experiments use the cotyledons of rapid-cycling fast plants (RCBr). However, the petioles were attached and shoot regeneration comes from the petiole end (site of meristematic tissues). No shoot regeneration was observed on media containing varying concentrations of BA and NAA when the petiole was removed and all edges were cut (data not shown; Teo et al. 1997). This may indicate that the sites

Table 4 Effects of age on shoot regeneration from cotyledonary explants of rapid-cycling *B. rapa*

Day (age)	Mean percentage (%) of explants producing shoots	Mean number of shoots per responding explant
3	47.5 ± 10.6 a	10.3 ± 7.1 a
4	44.5 ± 2.1 a	10.2 ± 1.8 a
5	18.0 ± 8.5 b	7.2 ± 4.9 a
6	20.0 ± 5.7 b	6.5 ± 5.1 a

Six, five, four, and three-day-old explants were excised from the proximal end of the cotyledon and placed adaxial side up on SRM 2 containing 1.5 mg/L TDZ, 0.5 mg/L NAA, and 5.0 mg/L AgNO3. Explants were observed every 2 weeks for 6 weeks for shoot production. Means with the same letter are not statistically significant at $P \leq 0.05$

of shoot regeneration in previous studies were from the attached petioles and not the cotyledons themselves.

TDZ has been documented to produce adventitious shoots and to increase shoot regeneration in recalcitrant species (including the Brassicas) when compared to other cytokinins such as BA (Cardoza and Stewart 2004; Jonoubi et al. 2005; Guo et al. 2005). An increased production of fasciated shoots and clustered shoot clumps was observed with increases in TDZ and NAA concentrations, which is consistent with previous findings (Guo et al. 2005). Thus, media FPM K (0.3 mg/L TDZ and 1.0 mg/L NAA) and FPM O (3.0 mg/L TDZ and 1.0 mg/L NAA) were not used for further studies due to the production of many more fasciated shoots than FPM I (1.5 mg/L TDZ and 0.5 mg/L NAA: Table 1).

Silver nitrate is a known ethylene inhibitor and has been shown to increase shoot proliferation in other *Brassica* species when used with cytokinins such as TDZ (Eapen and George 1997; Chi et al. 1990). In the presence of $AgNO_3$, more single, unfasciated shoots were regenerated. However, increasing the concentration of $AgNO_3$ from 5 mg/L (SRM2) to 10 mg/L (SRM3) decreased the number of explants producing shoots (from 20.2 to 8.5). In addition, increasing $AgNO_3$ concentration from 10 mg/L (SRM3) to 20 mg/L (SRM 4) decreased shoot production by 10.2%, i.e. 14.7–4.5% (Table 2), which confirms previous research (Teo et al. 1997).

The orientation and position of explants have been shown to affect shoot regeneration rates (Tran Thanh Van 1974). Explants excised from the basal part (proximal end) of several species of orchids yielded more explants producing shoots than explants excised from the apical region (distal end). These explants also produced more shoots per explant than those excised from the distal end (Tran Thanh Van 1974).

Klimaszewska and Keller (1985) also found that the orientation of explants on medium affected regeneration rates. A regeneration rate of 40% was obtained when explants excised from internodes of *B. napus* sp. *olerifera*, were placed adaxial side up on the medium. The regeneration rate dropped to 13% when the adaxial side (upper epidermis) was touching the medium (Klimaszewska and Keller 1985). Polarity was also observed in cotyledonary and hypocotyls explants of rapid-cycling *B. oleracea* (Cheng et al. 2001). The results in this experiment (Table 3) support these previous findings.

Previous experiments on RCBr mainly used 3-day-old cotyledonary explants with attached petioles (Teo et al. 1997). Explants of this age yielded the best shoot regeneration rate (47.5%) in this experiment; however, they were too small and consequently, difficult to cut. If this protocol is to be transferrable to K-12 and undergraduate education, 4-day-old explants are a better choice as they are larger and give essentially the same shoot regeneration rate (44.5%: Table 4). This data was also consistent with another study that used RCBr with attached petioles. The shoot regeneration rate for 3 and 4-day-old explants was 20 and 13%, respectively, whilst both 5 and 7-day-old explants only yielded a low 3% regeneration rate (Teo et al. 1997).

The highest regeneration rate (without intact petioles) recovered from this experiment (44.5%) is rather low for a model plant. However, all previous studies using RCBr gave a maximum regeneration rate of 33% with intact petioles (pre-existing meristems; Teo et al. 1997). In addition, the Brassicas are very recalcitrant in tissue culture with regeneration rates dependent on genotype, type and age of explants, and use of media additives including ethylene inhibitors (Cardoza and Stewart 2004). Future experiments to increase regeneration rates will include use of other ethylene inhibitors such as silver thiosulfate and aminoethoxyvinylglycine; and use of media additives such as putrescine which has had a synergistic effect with ethlylene inhibitors in other Brassicas (Cardoza and Stewart 2004).

It is important to note that leaves were able to produce adventitious shoots, albeit at a lower regeneration rate than cotyledons (23% vs. 44.5%). It takes about 9 days for the plants to produce leaves that are large enough to be excised. Future experiments will include the development of media for efficient regeneration of shoots from leaf explants.

In all shoot regeneration experiments, few individual shoots elongated on medium containing TDZ, NAA and $AgNO_3$. A basal medium with a low concentration of NAA was necessary for shoot elongation; some rooting was observed in this medium, however, most plants rooted on a hormone-free medium. Roots developed in 3 weeks and in vitro flowers were observed in most plants. In vitro flowering has been observed in many other crops including rapid-cycling *B. napus* (Koh and Loh 2000) and would not be unusual in RCBr, as it produces flowers in 2 weeks in vivo (Williams and Hill 1986). This would make this system ideal for the study of in vitro flower development for K-12 and undergraduate education.

It is recommended that the best medium for adventitious shoot regeneration from cotyledonary explants of rapid-cycling *Brassica rapa* is a MS based medium containing 1.5 mg/L TDZ, 0.5 mg/L NAA, and 5.0 mg/L $AgNO_3$. The explants should be excised from the portion of 4-day-old cotyledons that is proximal (closest) to, but not including, the petiole and should be placed on the medium adaxial side up for 4–6 weeks. These shoots should be transferred to elongation medium containing NAA for 2–3 weeks and then to hormone free medium for rooting within 2 weeks. This is the first report of efficient adventitious shoot regeneration from cotyledonary explants of rapid-cycling *B. rapa*.

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