BRIEF COMMUNICATION

In vitro multiplication of heavy metals hyperaccumulator *Thlaspi caerulescens*

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Abstract

A micropropagation protocol through multiple shoot formation was developed for *Thlaspi caerulescens* L., one of the most important heavy metals hyperaccumulator plants. *In vitro* seed-derived young seedlings were used for the initiation of multiple shoots on Murashige and Skoog (MS) medium with combinations of benzylaminopurine (BA; 0.5 - 1.0 mg dm^{-3}), naphthaleneacetic acid (NAA; 0 - 0.2 mg dm^{-3}), gibberellic acid (GA_3; 0 - 1.0 mg dm^{-3}) and riboflavin (0 - 3.0 mg dm^{-3}). The maximum number of shoots was developed on medium containing 1.0 mg dm^{-3} BA and 0.2 mg dm^{-3} NAA. GA_3 (0.5 mg dm^{-3}) in combination with BA significantly increased shoot length. In view of shoot numbers, shoot length and further rooting rate, the best combination was 1.0 mg dm^{-3} BA + 0.5 mg dm^{-3} GA_3 + 1.0 mg dm^{-3} riboflavin. Well-developed shoots (35 - 50 mm) were successfully rooted at approximately 95 % on MS medium containing 20 g dm^{-3} sucrose, 8 g dm^{-3} agar and 1.0 mg dm^{-3} indolebutyric acid. Almost all *in vitro* plantlets survived when transferred to pots.

Additional key words: micropropagation, growth regulators, riboflavin.

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Thlaspi caerulescens is known as a hyperaccumulator occurring in natural or contaminated soils with zinc, lead and cadmium (Baker *et al*. 1989). Its ability to uptake and accumulate large amounts of zinc and cadmium in their shoots (30 mg g^{-1} Zn and 10 mg g^{-1} Cd) without toxic effects make this plant an effective phytoremediator (Shah and Nongkynrih 2007). Conventional propagation of *T. caerulescens* from seed is slow and time consuming (Chaney *et al*. 1997). Therefore, an efficient *in vitro* propagation technique might provide an alternative means of producing large numbers of plantlets for further cultivation in metal-contaminated regions. Somatic hybrids between the zinc hyperaccumulator *T. caerulescens* and *B. napus* were produced successfully and indicated that that transfer of the trait for metal hyperaccumulation in plants is possible through somatic hybridization (Brewer *et al*. 1999). To date there are no reports on the micropropagation of *T. caerulescens*. Here, we report an efficient protocol for *in vitro* multiplication through shoot proliferation from seed-derived young seedling followed by the optimized conditions for acclimatization and transfer into greenhouse.

Seeds of *Thlaspi caerulescens* L. were supplied by Dr. Henk Schat (Institute of Ecological Sciences, Amsterdam, The Netherlands) and Dr. Mark Aarts (Wageningen University, Wageningen, The Netherlands) in October 2004 and then store at -20 °C until use. The seeds were first treated with 70 % ethanol (1 min) and then immersed in 0.1 % mercuric chloride (10 min), rinsed five times with sterile distilled water and then germinated on one-half strength MS medium supplemented with 30 g dm^{-3} sucrose and 7 g dm^{-3} agar (medium 1). The shoots (20 - 30 mm) were excised after 15 d and then transferred to MS medium containing

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Abbreviations: BA - benzylaminopurine, GA3 - gibberellic acid; IBA - indolebutyric acid; LH - lactalbumin hydrolysate; NAA - naphthaleneacetic acid; PGR - plant growth regulators.
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30 g dm^{-3} sucrose, 7 g dm^{-3} agar, 300 mg dm^{-3} lactalbumin hydrolysate (LH) supplemented with 1 mg dm^{-3} benzylaminopurine (BA) for initiation of multiple shoots (medium 2). Multiple shoots from medium 2 were further propagated on the same medium supplemented with BA (0 - 1.0 mg dm^{-3}), naphthaleneacetic acid (NAA; 0 - 0.2 mg dm^{-3}) and gibberellic acid (GA_3; 0 - 1.0 mg dm^{-3}). To determine if the riboflavin could stimulate plant growth and improve the shoot biomass, multiple shoot explants (4 - 5 shoots, 25 - 30 mm) were excised from their bases and placed vertically on MS agar medium containing 30 g dm^{-3} sucrose, 300 mg dm^{-3} LH, 1.0 mg dm^{-3} BA, 0.5 mg dm^{-3} GA_3 supplemented with 0, 0.5, 1.0, 3.0 mg dm^{-3} riboflavin. Elongated shoot explants (cluster of 4 - 6 shoots) were inoculated onto MS medium containing 10 g dm^{-3} sucrose and 7 g dm^{-3} agar, supplemented with different concentration of either indolebutyric acid (IBA; 0 - 1.0 mg dm^{-3}) or NAA (0 - 1.0 mg dm^{-3}) for root induction. Plantlets with well-developed shoots (60 - 80 mm) with roots (20 - 30 mm) were removed from the flasks and washed gently in running tap water to remove excess agar medium and sucrose traces to discourage infection by fungal contaminants. They were then transferred to 15-cm-diameter plastic pots containing a mixture of soil and *Vermiculite* (1:1 ratio) and covered with polythene bags for 5 - 7 d to prevent excessive water loss. After transfer, the plants were placed in a greenhouse and watered twice a week. The survival rate was recorded once a week for 2 months. All the media used in the investigation were adjusted to pH 5.6 before autoclaving at 1.4 kg cm^{-2} (121 °C) for 18 min. About 40 cm^3 of medium was dispensed in 100-cm^3 flasks for tissue culture experiment with three replicates. Cultures were maintained at 22 ± 3 °C under a 16-h photoperiod with a photosynthetic photon flux density (PPFD) of 45 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps. Cultures were regularly transferred onto fresh medium every 4 weeks. The shoot number (> 1.0 cm long) and shoot length per culture were counted at the end of subculture intervals. Each treatment had 3 replicates containing 8 explants for adventitious shoot regeneration and micropropagation experiments.

Mature seeds of *T. caerulescens* germinated (90 %) and grew into young seedling in 2 weeks on medium 1

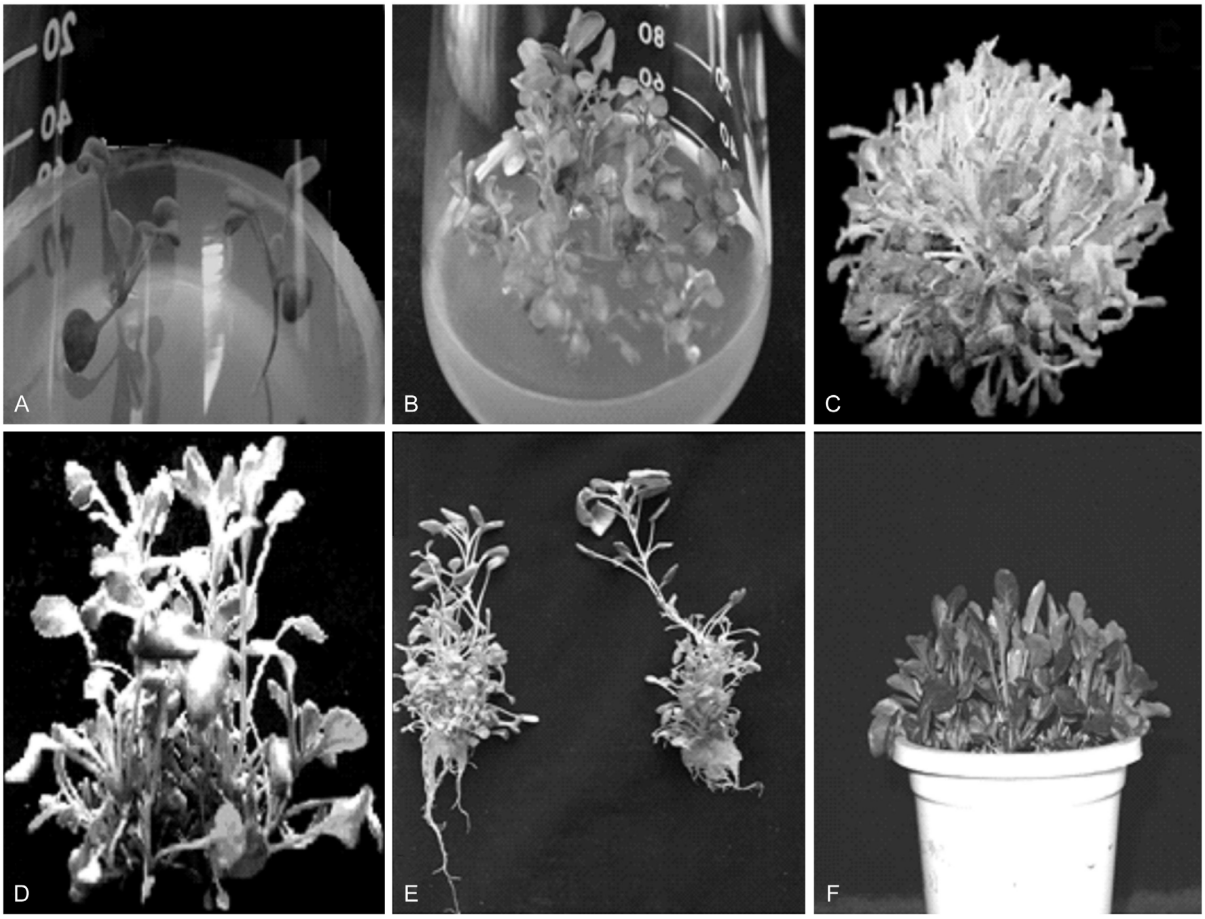

Fig. 1. Plant regeneration from *in vitro* seed-derived young seedlings of *T. caerulescens*: *A* - seeds germination on one-half strength MS medium, *B* - *in vitro* shoots formed on MS medium with 1 mg dm^{-3} BA in the initial culture, *C,D* - multiple shoots formed on MS medium with 1.0 mg dm^{-3} BA and 0.2 mg dm^{-3} NAA (C) or 1.0 mg dm^{-3} BA, 0.5 mg dm^{-3} GA_3 and 1.0 mg dm^{-3} riboflavin (*D*), *E* - plantlet showing roots induced on MS medium containing 1.5 mg dm^{-3} IBA, *F* - regenerated plants in the soil.

Table 1. Effects of growth regulators on *in vitro* shoot multiplication of *T. caerulescens* after 4 weeks of culture.

BA [mg dm^{-3}]	NAA [mg dm^{-3}]	GA_3 [mg dm^{-3}]	Number of shoots [expl.$^{-1}$]	Shoot length [mm]
0	0	0	9.5 ± 2.4	36.0 ± 4.8
0.5	0	0	70.0 ± 3.2	26.5 ± 2.3
1.0	0	0	78.0 ± 3.2	23.0 ± 1.7
1.0	0.1	0	86.5 ± 3.5	25.0 ± 1.2
1.0	0.2	0	92.0 ± 5.4	32.1 ± 1.7
1.0	0	0.5	71.6 ± 2.8	39.2 ± 1.5
1.0	0	1.0	67.4 ± 1.5	37.9 ± 3.2

Table 2. Effects of riboflavin on *in vitro* shoot multiplication of *T. caerulescens* on agar MS medium containing 1.0 mg dm^{-3} BA and 0.5 mg dm^{-3} GA_3 after 4 weeks of culture.

Riboflavin [mg dm^{-3}]	Number of shoots [expl.$^{-1}$]	Shoot length [mm]
0	71.6 ± 2.8	39.2 ± 1.5
0.5	75.2 ± 1.8	43.1 ± 0.9
1.0	77.7 ± 1.9	46.2 ± 0.7
3.0	76.4 ± 2.4	37.3 ± 1.4

Table 3. Effects of NAA and IBA on *in vitro* rooting of shoots ranging a length between 35 - 50 mm of *T. caerulescens* on agar MS medium after 4 weeks of culture.

Auxin	Conc. [mg dm^{-3}]	Rooting [%]	Number of roots [expl.$^{-1}$]	Root length [mm]
0	0	70	13 ± 1.1	43 ± 1.2
IBA	0.5	90	23 ± 0.5	47 ± 0.8
	1.0	95	20 ± 0.2	54 ± 0.9
NAA	0.5	68	14 ± 0.5	20 ± 1.8
	1.0	36	6 ± 0.1	15 ± 0.2

(Fig. 1*A*). On an average, 14 shoots were formed per original explant in the initial cultures grown on medium 2 (Fig. 1*B*). These shoots appeared to be proliferated directly from node *via* the axillary branching of buds from the young seedling explants and were maintained as an individual clone for subsequent culture. Regeneration was observed on all media, with total numbers of regenerants ranging from 9.5 to 92 per explant (Table 1). A regeneration rate of 9.5 was found on PGR-free medium. BA was very effective in the shoot proliferation, while decreased significantly the shoot length which influenced the subsequent rooting. In this experiment, the greatest number of shoots was formed on the medium containing 1.0 mg dm^{-3} BA and 0.2 mg dm^{-3} NAA (Fig. 1*C*). It was noteworthy that mean shoot length in the experiment is no more than 35 mm and the rooting rate was low. Therefore, we try to modify the phytohormone combinations and mean shoot length increased significantly when GA_3 was added to BA-containing medium (Table 1). Besides others, GA_3 promotes stem elongation and accelerate shoot growth (Biemelt *et al*. 2004). In the present study, addition of GA_3 to the BA containing media increased shoot length but decreased shoot multiplication (Table 1). This result was consistent with the findings of Conover and Litz (1978) and Karim *et al*. (2003) and was contrary to the report by Wilna de Winnar (1988) that GA_3 with BA-NAA formulation stimulated both proliferation and elongation of stem. A similar propagation protocol with combinations of BA and GA_3 has also been reported for apple rootstock (Kaushal *et al*. 2005) and *O. mungo* (Jose and Satheeshkumar 2004). It was also notable that effect of combined 1.0 mg dm^{-3} BA, 0.5 mg dm^{-3} GA_3 and 1.0 mg dm^{-3} riboflavin enhanced the growth and exceeded the shoot numbers obtained with other media (Table 2, Fig. 1*D*). Regenerated shoots were excised from explants and transferred to agar MS medium containing 0 - 2.0 mg dm^{-3} of one of the auxins IBA or NAA. Although rooting frequency as well as average root number and length differed, adventitious roots formed in all treatments. Shoots rooted *in vitro* in the PGR-free culture medium within 4 weeks of culturing with a frequency of 70 %. Well-developed shoots (35 - 50 mm) were successfully rooted at approximately 95 % on MS medium containing 20 g dm^{-3} surcose, 8 g dm^{-3} agar and 1.0 mg dm^{-3} IBA (Fig. 1*E*). The rooted plantlets were replanted into the soil and *Vermiculite* (1:1) where well-developed root system was formed during 2 - 3 weeks. The clone survival rate was about 95 %. After acclimatization, plantlets have grown actively in the greenhouse with normal leaf and shoot morphology (Fig. 1*F*). The *in vitro*-derived plants did not show any detectable variation in morphology or heavy-metal tolerance ability (data not shown). Based on these observations, we conclude that our protocol for the rapid propagation of *T. caerulescens* is feasible for the mass production of plantlets for further field cultivation in metal-contaminated regions.

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Plant breeding is an activity that is closely related to history and people's livelihoods, even if they do not realise it! The concise encyclopedia presented here calls the attention of the public to this fact. The author, a professor of cytogenetics and plant breeding, recognizes the need for such a comprehensive document, which depicts the long and really exciting story of how plant breeding activities have developed alongside civilization since the first steps towards the transformation of wild plants into crops took place ten or so thousands of years ago.

The main content of the encyclopedia is well arranged into six chapters, followed by some additional information (Notes, Glossary, Bibliography and Index).

In Chapter 1 (Introduction) the reader is given an explanation of the way crop plants appeared on the earth. A comprehensive Chapter 2 (10 000 Years of Crop Improvement) follows, telling the history of plant breeding. Despite the fact that the greatest achievements of plant breeding occurred after the 'scientific approach' was introduced, following the re-discovery of Mendel's laws, we learn about evidence of systematic approaches, and even about artificial pollination, in ancient times. Reading this chapter provides an overview concerning the contributions of different human cultures to the breeding process.

Chapter 3 (Mendel´s Contribution to Inheritance and Breeding) is the largest part of the book. At the beginning of 20th century the start of the new scientific discipline called "genetics" brought about a rapid development and diversification of the methodical approaches to plant breeding. In this period the breeder´s interests became interwoven with scientific views and terms like "green revolution" appeared. The utilization of mathematics and computation techniques accelerated immensely the progress in understanding, and in the application, of genetic rules.

Chapter 4 (Biotechnology, Genetic Engineering, and Plant Improvement) presents a concise compendium of the newest approaches in genetics and their application to plant breeding. Twenty-one *in vitro* techniques are listed that are utilized in plant breeding and in the production, rescue and conservation of plant materials. Many molecular methods based on the PCR technique have been developed and applied as "Marker-Assisted Selection", and transgenic crop plants have been obtained following "plant transformation".

Chapter 5 (Intellectual Property Rights, Plant Variety Protection, and Patenting) gives a brief but useful overview concerning the legal context of modern breeding activities as considered in different countries. Legal conditions have become a very important background for further development, and particularly in the application of advanced genetic techniques.

Chapter 6 (In the Service of CERES – a Gallery of Breeders, Geneticists, and Persons Associated with Crop Improvement and Plant Breeding) presents a list of many people connected with the history of plant breeding.

The book offers a very pleasant read and a nice overview of the history of plant breeding to any person interested in this subject: student, teacher, breeder or scientist.

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