BioTechnologia vol. 93(2) • pp. 97-101 • 2012
Journal of Biotechnology, Computational Biology and Bionanotechnology RESEARCH PAPER

In vitro multiplication and acclimatization of *Biscutella laevigata* (Brassicaceae) to cultivation in greenhouse conditions

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Abstract

The suitability of *in vitro* culture technique for vegetative propagation of *Biscutella laevigata* calamine ecotype was investigated. Various organic additives were tested as medium supplements: squash obtained from fleshy pineapple fructification, liquid endosperm of coconut, and a conditioned medium acquired from the culture of green algae. Exploited plant-derived biostimulators along with growth regulators included in the propagation medium, improved *B. laevigata* multiplication *in vitro*. The highest micropropagation coefficient, exceeding three adventitious rosettes from one maternal microcutting, was obtained in the medium containing 10 ml · l^{-1} pineapple pulp during a 6-week-long culture. Microrosettes were rooted *in vitro* in a hormone-free, modified propagation medium, with macro- and microelements accounting for one third of the initial concentration. Acclimatization to *ex vitro* environment was conducted both in growth chamber and greenhouse conditions. Plants cultivated in the greenhouse bloomed and set seed. The results of the experiment allowed determination of the most suitable conditions required for culture initiation and successful development of a protocol for *in vitro* propagation of *Biscutella laevigata* ecotype obtained from the Olkusz district.

Key words: *Biscutella laevigata,* biostimulators, *in vitro*, micropropagation, cultivation

Abbreviations

2iP	– 2-isopentenyladenine	PGRs	– plant growth regulators
NAA	– 1-naphtaleneacetic acid	PPFD	– photosynthetic photon flux density

Introduction

Biscutella laevigata L. *sensu lato* (Brassicaceae) is a polymorphic species which has developed different ecotypes, and is consequently recorded in rocky, often alpine, European stands, metal enriched soils, and anthropogenic substrates, such as waste heaps polluted with heavy metals (Tremetsberger et al., 2002; Armiraglio et al., 2005; Mikuška, 2004; Parisod and Joost, 2010). It is a beautiful perennial herb with simple leaves gathered in a basal rosette, and a leaved branched inflorescence stalk. Numerous bright-yellow flowers and unique in shape siliques increase the aesthetic value of this Brassicaceae representative. In Poland, *B. laevigata* is quite uncommon, as it has only been described from stands located in the Tatra Mountains (Jasiewicz, 1985; Mirek and Piękoś-Mirkowa, 2003) and in the Zn-Pb ore-mining region as an endemic metallophyte, typical of calamine flora from the Olkusz district (Godzik 1993; Kowolik et al., 2010; Szarek-Łukaszewska and Grodzińska, 2011).

Development of alternative propagation methods that utilize *in vitro* techniques, allows the creation of other means of protecting valuable plant species efficiently at the population as well as the ecotype level (Rybczyński et al., 2004; Rybczyński and Mikuła, 2006). The possibility of raising the propagation coefficient using such techniques will certainly contribute towards effortless obtaining of numerous specimens, ready to be used as uniform plant material in the reclamation of waste heaps polluted with lead and cadmium, in phytoremediation, or in naturalistic gardens established in polluted sites (laCoste et al., 1999; Orłowska et al., 2002; Hanus-Fajerska et al., 2010; Hanus-Fajerska, 2011). Due to the above-mentioned reasons, the aim of our experiments was to determine convenient conditions for culture initiation, and to elaborate the protocol of *in vitro*

propagation of *Biscutella laevigata* calamine ecotype from the Olkusz district.

Material and methods

Donor plant material

Donor material to initiate *in vitro* experiments were seeds of *Biscutella laevigata* species belonging to the calamine population from the Olkusz ore-bearing region, located in the south-eastern part of the Śląsko-Krakowska Upland, Poland (50°17′N, 19°29′E). The siliques were collected thrice during the vegetation period of 2010 (23rd May, 13th June, 27th June). Seed samples obtained during every term were divided in 30 sub-samples. Each comprised 10 seeds that were subsequently surface decontaminated using 70% ethanol followed by treatment with 0.1% mercuric chloride for 3 min. After three washes in sterile distilled water, seeds from each sample were placed in a separate container onto MS medium (Murashige and Skoog, 1962) without plant growth regulators (PGRs). Culture containers of 200 cm^3 capacity were used, with 50 cm^3 of culture medium solidified with 0.8% Difco agar. In the first stage of the experiment, the seed germination ability under *in vitro* conditions was determined. Germination was carried out in an air-conditioned chamber irradiated with white light (40 $\mu mol/m^2/s^1$ PPFD; Fluora-Osram 36W/77 lamps), at constant temperature (20 ± 2°C), and under 12-h photoperiod. The experiment was conducted in three replications, in each 100 seeds were used from every silique-harvesting term. The assessment of germination ability was performed 21 days after the beginning of the experiment.

Scheme of experimental set

Seedlings obtained in aseptic conditions and deprived of roots were used as primary explants. Explants were placed on a modified MS medium, supplemented with 25 g/l sucrose, 0.6 g/l calcium gluconate, 0.6 g/l activated charcoal, 1.0 mg/l 2iP, 0.1 mg/l NAA, and solidified with 0.7 g/l Difco Bacto agar. The pH of the medium was adjusted to 5.8. This medium was regarded as the control medium in subsequent experiments (further described as control). From this stage of experiment, Erlenmeyer flasks (250 cm^3 capacity), containing 50 cm^3 of medium were used until the completion of the experiment. The culture environment was maintained at 24 ± C2° during the day, and 20 ± 2° C during the night, with photoperiod 16h/8h, and 80 μmol/m/s PPDF at the culture level, secured with cool white fluorescent lamps. The relative humidity of air in the growth chamber fluctuated from 70-72%. The explants were excised from 30 well-shaped seedlings, and the experiment was repeated three times, each time in five replications (culture container with six explants). Passages onto the fresh medium of the same composition were performed every 21 days.

When stabilized cultures were obtained, another experimental setup was established to investigate the impact of additional plant-derived material, rich in organic compounds, on the propagation coefficient of *B. laevigata* microrosettes. Organic supplements were procured from squash obtained from fleshy *Ananas comosus* (Stickm.) Merr. (Bromeliaceae) fructification (further described as pineapple pulp), liquid endosperm from *Cocos nucifera* L. (Arecales) drupe (further described as coconut water), and spent medium after the culture of *Desmodesmus subspicatus* (R. Chodat) E. Hegewald & A. Schmidt (Chlorophyceae), further described as CM1 and CM2 depending on the exact concentration of the conditioned medium used. The spent medium was kindly provided by Professor Z. Tukaj and Dr. K. Grabski, from the Department of Plant Physiology, University of Gdańsk, Poland. The control MS medium, supplemented with 1.0 mg/l 2iP, 0.1 mg/l NAA, was enriched with one of the following organic additives: 10 ml/l of pineapple pulp, 10 ml/l of coconut water, and 20% (CM1) or 50% (v/v) (CM2) of the conditioned medium obtained from the culture of *D. subspicatus*. Two additional control treatments were also performed: (i) "control treatment" – with the same medium as the one used during stabilization of *B. laevigata* cultures, and (ii) "subcontrol treatment" – with control MS medium deprived of PGRs, but supplemented respectively with 10/ml/l of pineapple pulp (pineapple pulp 0), 10 ml/l of coconut water (coconut water 0), and with 20% (CM1-0) or 50% (CM2-0) of the conditioned medium. The culture environment was unaltered, and similar to the previous experimental setup, passages onto a fresh medium of the same composition were conducted every 21 days. Adventitiously regenerated microrosettes were not separated from the maternal one during the passage. The experiments were carried out thrice for six weeks, and each treatment was

replicated five times. The culture container with six explants constituted a replicate. Macroscopic observations were done every second day, and the multiplication coefficient, regarded as the number of adventitious rosettes obtained from the maternal one, was evaluated 42 days after the commencement of the experiment. Afterwards, the obtained rosettes were rooted *in vitro* in thrice-diluted control medium without PGRs. The rooted rosettes were transplanted to ceramic flower pots 90 mm in diameter with autoclaved potting mixture of perlite, sand and horticultural soil (1:1:1 v/v), and maintained for 28 days in a controlled growth chamber at the same conditions as during the micropropagation stage with the exception of relative humidity, maintained at 50%. Next, the plants were placed in a greenhouse, where they were being gradually transplanted to larger containers filled with the mixture of perlite, sand, and post-flotation wastes obtained in the process of zinc-lead ores enrichment (1:1:3 v/v).

The results appropriate to statistical analysis were subjected to one way STATISTICA 9.0 ANOVA (StatSoft Inc.), and a posteriori Fisher's test was used to determine the significance of differences between the studied objects, with the significance level of $\alpha = 0.05$.

Results and discussion

In the present study, seed samples were obtained from *Biscutella laevigata* metalliferous ecotype that grows in post-mining areas (Fig.1A). Numerous sterilizing agents were primarily tested (data not shown), and the most effective surface decontamination of seeds was achieved using 0.1% solution of $HgCl_2$. The greatest number of properly shaped seedlings that could easily develop to plantlets was obtained as a result of germination of seeds sampled from the latest term, i.e. 27 June. Such seeds germinated on average at 65.5% (the mean value from three replications), whereas the germination ability of the seeds sampled earlier (on 23 May and13 June) was low and accounted for 13.4% and 26.8%, respectively. Seedlings obtained from those samples were not used in further experiments.

From the seedlings gained in aseptic conditions, stabilized cultures were readily obtained (Fig. 1B) and within nine weeks sufficient material was obtained to commence the next experimental setup. It was designed to investigate the impact of the additional plant-derived material, rich in organic compounds, on the micropropagation coefficient. As a result of the conducted experiments, well-rooted *B. laevigata* rosettes were obtained (Fig. 1C – Fig. 1D), which after being transplanted to substratum (Fig. 1E) successfully acclimatized to greenhouse conditions (Fig. 1F) and were cultivated until flowering (Fig. 1G) and seed setting.

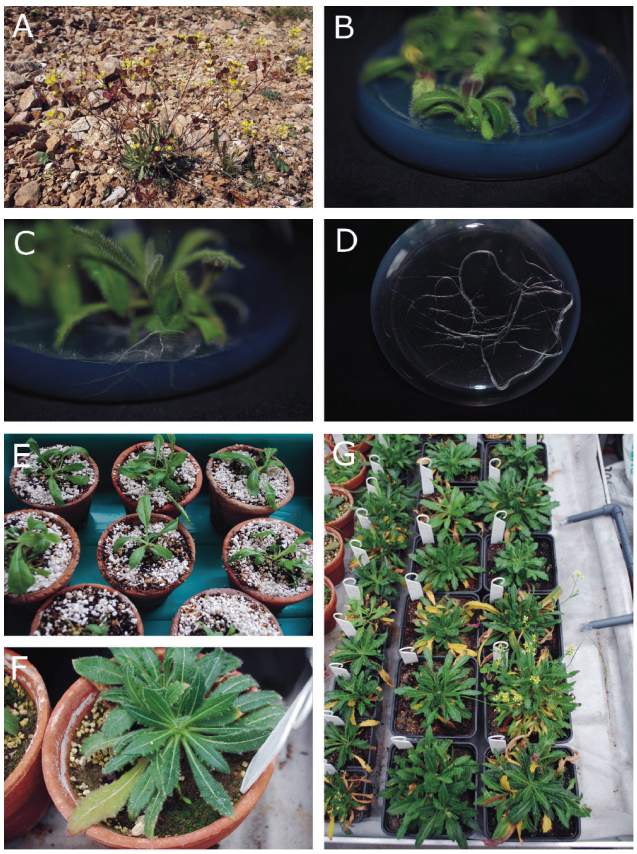

Fig. 1. *Biscutella laevigata* ecotype from the Olkusz Zinc-lead Ore District: A) plant habit; Macroscopic record of the micropropagation phase: B) proliferating culture on a modified MS medium supplemented with 1.0 mg/l 2iP, 0.1 mg/l NAA C) rooting on a diluted MS without plant regulators, D) aspect of the rooting phase, E) obtained plantlets transplanted to the mixture of perlite, sand and horticultural soil (1:1:1 v/v) during acclimatization in a controlled growth chamber, F) juvenile plants transferred to the greenhouse, G) cultivation of plants under greenhouse conditions

A positive influence of the applied organic supplements on *B. laevigata* micropropagation was observed in the media containing PGRs (Fig. 2). The highest number of adventitious rosettes was obtained on a medium enriched with 10 ml/l pineapple pulp, where the mean micropropagation coefficient (MC) reached 3.3. A slightly lower efficiency of rosette multiplication was noted in the

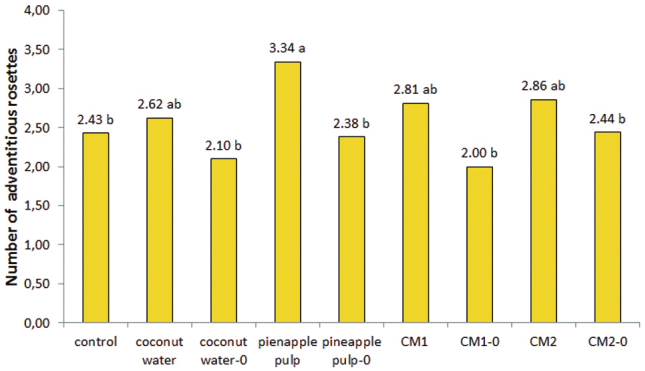

Fig. 2. Impact of additional plant-derived material, rich in organic compounds, on the number of adventitious regenerations from *Biscutella laevigata* maternal microrosette. Control MS medium supplemented only with: 10 ml/l of pineapple pulp – pineapple pulp 0, 10 ml/l of coconut water – coconut water 0 with 20% (CM1-0) or 50% (CM2-0) of the conditioned medium respectively

media containing a conditioned medium in both tested concentrations (MC = 2.8), and coconut water (MC = 2.6). Surprisingly, in the media without synthetic growth regulators, but supplemented with any of the tested natural ingredients, the formation of adventitious rosettes was equal to that in the control medium containing synthetic PGRs. This indicates a possibility of reducing the use of synthetic auxins and cytokinins in the clonal propagation of the examined species.

There is an intensive search for new kinds of natural substances, called biostimulators going on nowadays in sustainable agriculture, and especially in horticulture. Those substances, when applied on certain plants, considerably enhance their vigor and productivity (Przybysz et al. 2010; Lisiecka et al. 2011; Woropaj-Janczak et al., 2011). Similarly, in *in vitro* culture, the health and appearance of plantlets can be improved, and the micropropagation coefficient may increase. The efficacy of an additional medium supplementation have already been proven in cultures of several plant species (Mechanda et al., 2003; Peixe et al., 2007; Agampodi and Jayawardena, 2009; Shrivastava and Banerjee, 2009).

It has been found that concurrency of additional supplements, such as plant tissues and exudates, with phytohormones included in the proliferation medium, promotes the formation of adventitious rosettes in *Biscutella laevigata*. It provides a background for elaboration of an eco-friendly micropropagation protocol, in which the use of synthetic growth regulators could be at least partially substituted with natural organic additives of biostimulatory character.

Summary

An effective micropropagation protocol of *Biscutella laevigata* calamine ecotype from the Olkusz district has been developed. An effortless way to start *in vitro* cultures of that metallophyte has been established by obtaining primary explants from seedlings previously acquired in aseptic conditions, provided that the term of seed sampling is optimized. The microrosettes multiplied *in vitro* after rooting can be effectively acclimatized to *ex vitro* conditions, and the cultivation of the plants can be further conducted under greenhouse conditions until flowering and seed setting.

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