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Short Communication

Zeatin and TDZ-induced Shoot Proliferation and Use of Bioreactor in Clonal Propagation of Medicinal Herb, Roseroot (*Rhodiola rosea* L)

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A procedure for *in vitro* propagation of roseroots (*Rhodiola rosea* L), a medicinal plant, was developed using a RITA bioreactor system containing liquid medium, combined with a gelled medium. Wild roseroot clones: 'RC1', 'RC2' and 'RC3' were established on a basal medium (BM) from *in vitro*-germinated seedlings on half-strength Murashige and Skoog (MS) salts. TDZ at 2-4 μM supported shoot proliferation but inhibited shoot elongation of 'RC1' shoots on gelled medium. Clones differed significantly with respect to multiplication rate with 'RC1' producing the most shoots per explant on gelled BM with 2 μM zeatin. In a bioreactor system, TDZ supported rapid shoot proliferation at lower concentration (0.5 μM) but induced hyperhydricity at more than 0.5 μM. Bioreactor-multiplied hyperhydric shoots of all clones when transferred to gelled medium containing 1-2 μM zeatin produced normal shoots within 4 wk of culture. Shoots were rooted *in vitro* on BM void of growth regulators. Almost all (90 to 95%) *in vitro* plantlets survived when transferred to potting medium.

Key words: bioreactor, cytokinins, medicinal herb, micropropagation.

The roseroot (*Rhodiola rosea* L), also called golden root or arctic root, is a dioecious, perennial herb of family *Crassulaceae*. The yellow-flowered taxon of roseroot is found in the mountain regions of Central and Northern Europe, Russia and in the east coastal regions of North America at altitudes between 1000 and 5000 m above sea level. Historically, it is used as an adaptogen in Russia, northern Europe and in China as a traditional herbal medicine, and is valued for its ability to enhance human resistance to stress or fatigue, and promote longevity (1).

In vitro propagation of selected germplasm can potentially multiply collected plants more rapidly than traditional propagation methods. Although there are some reports of *in vitro* culture of roseroot (2-6), none is available using bioreactor for mass propagation. Bioreactors are self-contained, sterile environments which capitalize on liquid nutrient or liquid/air inflow and outflow systems, designed for intensive culture and control over microenvironmental conditions (aeration, agitation, dissolved oxygen, etc.) (7). The objective of the current study was to determine the influence of thidiazuron (TDZ) and zeatin in roseroot *in vitro* culture and to focus on the development of a cost-effective method for commercial micropropagation of wild roseroot using a combination of gelled medium and bioreactor system.

Open pollinated seeds obtained from wild grown plants of Mikkeli, Finland (61°41′15″ N; 27°16′25″ E) were disinfected following the method developed by Debnath (8) and placed on germinating medium containing half-strength MS (9) salts and 20 g l^{-1} sucrose and gelled with 7.5 g l^{-1} agar with the pH adjusted to 5.7 prior to sterilizing by autoclaving at 121°C for 15 min. Seedlings were grown aseptically, each in a 16 × 150 mm test tube containing 7 ml medium at 22 ± 2°C under a 16-h photoperiod with a photosynthetic photon flux density (PPFD) of 30 μmol m^{-2} s^{-1} at the culture level provided by cool-white fluorescent lamps. Seed germination took place within 6-8 days of culture.

Three clones ('RC1', 'RC2' and 'RC2') were developed from the vegetative shoots obtained *in vitro* from three individual seedlings, selected based on good vigour under *in vitro* condition after 8 wk of germination, and were multiplied and maintained by sub-culturing of shoot tips

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Abbreviations: BM, basal medium; PPFD, photosynthetic photon flux density; MS, murashge and skoog; TDZ, thidiazuron; TIB, temporary immersion bioreactor.

(0.8-1 cm long) in 175 ml glass baby food jars containing 35 ml basal medium [BM, three-quarter macro-salts and micro-salts of Debnath and McRae's (10) shoot proliferation medium D] supplemented with 2 μM zeatin and 25 g l^{-1} sucrose, gelled with 3.5 g l^{-1} Sigma A 1296 agar and 1.25 g l^{-1} Gelrite. Culture jars were maintained at 20 ± 2°C under a 16 h photoperiod with a PPFD of 30 μmol m^{-2} s^{-1} at the culture level provided by cool white fluorescent lamps. For each experiment, explants were grown for another 8 wk on BM that contained no plant growth regulators (PGRs) to minimize the effect of PGR.

To study the effects of TDZ and zeatin for shoot proliferation on gelled medium (experiment 1), shoot tips (0.8-1 cm long), collected from 8 wk-old shoots of 'RC1', were bulked and transferred at random in 175 ml glass baby food jars containing 35 ml gelled BM supplemented with 0, 1, 2 or 4 μM TDZ or zeatin and 25 g l^{-1} sucrose. There were five jars for each treatment and each jar contained one explant. The experiment was repeated four times.

In the second experiment, the response of clones: 'RC1', 'RC2' and 'RC2' was studied over four sub-culture periods following the same procedure as was followed in the previous experiment. Sub-culturing was done every 8-wk intervals with the shoot tips (0.8-1 cm long) randomly selected from 8-wk old *in vitro*-grown shoots. Shoot tips were grown on BM with 2 μM zeatin. The experimental unit consisted of one explant in each jar, and there were six jars for each treatment. The experiment was replicated three times.

The third experiment was conducted with 'RC1' to study shoot proliferation in a temporary immersion bioreactor (TIB) vessel [RITA® bioreactors (VITROPIC, Saint-Mathieu-de-Treìviers, France; 11)] containing 200 ml of liquid BM with 0, 0.5, 1 or 2 μM TDZ, over four sub-culture periods. Temporary immersion cultures were established with immersion of explants for 15 min every 4 h. Shoot tips (0.8-1 cm long), derived from PGR-free gelled BM, were transferred into bioreactor vessels and the cultures were maintained following the procedure of the previous experiments. Sub-culturing was done every 8-wk intervals over four periods with the randomly selected explants. There were three vessels for each treatment and each vessel contained eight explants. The experiment was conducted two times.

Eight-wk-old elongated shoots (1-1.5 cm long) of three clones obtained from bioreactor vessel containing liquid medium supplemented with 0.5 μM TDZ, were excised individually and cultured for 8 wk on BM that contained no PGR for rooting. Rooted shoots were rinsed free of tissue culture medium, and planted in 45 cell plug trays (cell diameter: 5.9 cm, cell depth: 15.1 cm) containing ProMix BX potting medium. Trays were placed in a humidity chamber with a vaporizer and acclimatized by gradually lowering the humidity over 2-3 wk (temperature 20 ± 2°C, humidity 95%, PPFD = 55 μmol m^{-2} s^{-1}, 16-h photoperiod). Hardened-off plants were transferred to 10.5 cm^2 plastic pots containing the same medium and maintained in the greenhouse (temperature 20 ± 2°C, humidity 85%, maximum PPFD = 90 μmol m^{-2} s^{-1}, 16-h photoperiod). Per cent survival was recorded at 8 wk after removal from tissue culture.

Surviving explants were measured for each treatment at 8 wk for number of shoots (> 1 cm long) per responding explant, shoot height (cm), number of leaves per shoot and shoot vigour. Vigour was determined by visual assessment, on a scale of 1 (strongly vitrified, necrotic and/or malformed shoots) to 8 (fully normal and healthy shoots with excellent vigour).

Data for all characters except shoot vigour were subjected to analysis of variance with the SAS statistical software package (Release 8.2, SAS Institute, Inc., Cary, N.C.). Shoot vigour was analyzed separately by categorical analysis (CATMOD procedure in SAS). In the third experiment, data for the control treatment (0 TDZ) were excluded from analysis because no explants responded after the first sub-culture.

Incubation for about 2 wk was required for shoots to emerge from pre-existing axillary buds which grew only a couple of centimetres in a 4 wk incubation period. Appearance of the white callus at the base of the explant indicated shoot establishment in the medium (Fig. 1A). Shoots appeared to be proliferated directly from the node via the axillary branching of buds from the original explants (Fig. 1B).

In the first experiment, cytokinin type and concentration affected shoot proliferation but not on shoot vigour. Increasing the concentration of TDZ or zeatin in the medium significantly ($P \leq 0.01$) increased the number of shoots per explant, but decreased shoot length and leaf number per shoot (Fig. 2 A-C). Shoot number per explant was better on BM containing TDZ compared to zeatin and no PGR, although shoot length, leaf number per shoot and shoot

vigour were better in zeatin-containing medium. While the number of shots per responding explant was greatest at 4 µM TDZ, shoot length and leaf number per shoot were best at 1 µM zeatin, and shoot vigour at 2 µM zeatin (Fig. 2).

Shoots of the clones 'RC1' did not elongate more than 1-2 cm during the 8 wk following culture initiation in BM containing 1-4 µM TDZ. Cultures were then transferred to BM containing 1 or 2 µM zeatin where shoots elongated within 4 weeks (data not shown). Shoots growing for more than 10 wk on media that contained 4 µM TDZ, occasionally produced hyperhydric adventitious shoot masses, which appeared to arise from dense calluses growing at the base of the shoots in the medium (Fig. 1C).

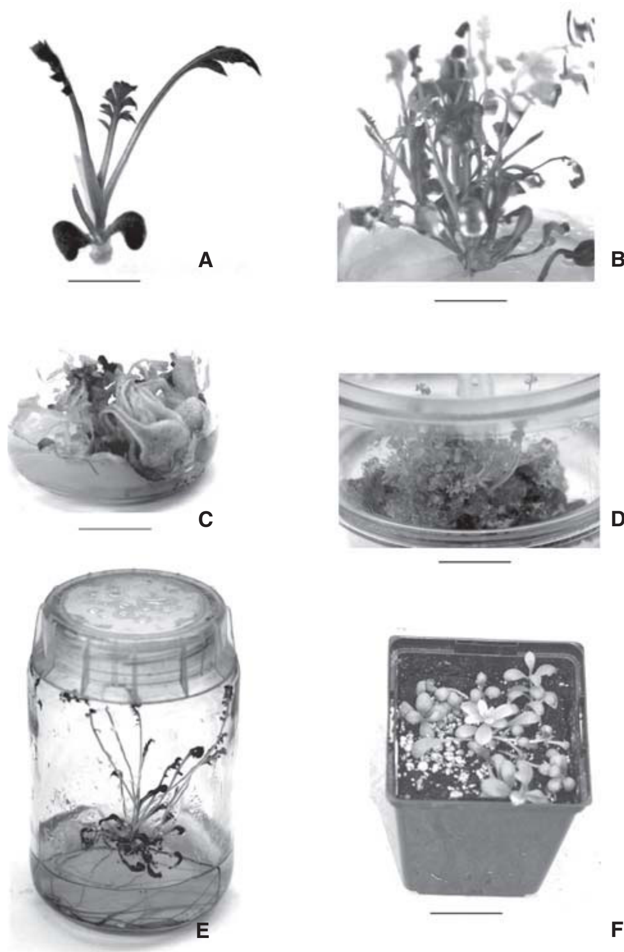

Fig. 1. *In vitro* shoot proliferation of roseroot clone 'RC1'. (**A**) Shoot proliferation after 4 wk on gelled basal medium (BM) supplemented with 2 µM zeatin. Bar = 1 cm. (**B**) Shoot proliferation after 8 wk on gelled BM supplemented with 2 µM zeatin. Bar = 2.5 cm. (**C**) Hyperhydric adventitious shoot regeneration after 12 wk on gelled BM supplemented with 4 µM TDZ. Bar = 2 cm. (**D**) shoot multiplication after 8 wk in a bioreactor vessel containing liquid BM supplemented with 0.5 µM TDZ. Bar = 2.5 cm. (**E**) *In vitro* rooting after 8 wk on gelled BM containing no plant growth regulator (PGR). Bar = 3 cm. (**F**) Greenhouse-grown micropropagated plant after 12 wk under *ex vitro* conditions. B = 6 cm.

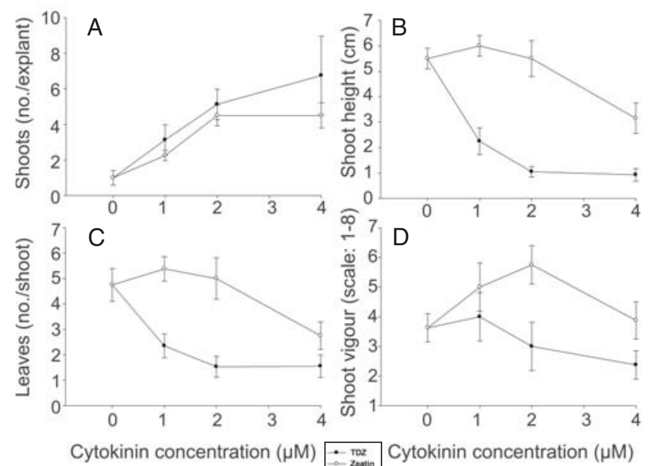

Fig. 2. The influence of varying concentrations of thidiazuron (TDZ) and zeatin on shoot proliferation of roseroot clone 'RC1' grown on gelled medium for 8 wk: (**A**) shoots (no./explant), (**B**) shoot height (cm), (**C**) leaves (no./shoot), and (**D**) shoot vigour (scored on a scale from 1 to 8, with the poorest shoot being 1 and 8 the best). Vertical bars indicate ±SD.

In experiment 2, Across all treatments, shoot number per explant and leaf number per shoot increased with sub-culturing up to the third and second sub-culture periods, respectively and then stayed the same. 'RC1' produced more vigorous and longer shoots with more leaves per shoot than those of 'RC2' and 'RC3' (data not shown).

Results of the third experiment revealed that axillary buds with multiple shoots developed at all TDZ concentrations in the first sub-culture. The number of shoots per explant, shoot height, leaf number per shoot and shoot vigour improved at 0.5 µM TDZ, but declined with higher concentration in the liquid medium (Fig. 3). Across all treatments, an average of 16 shoots per explant was obtained when 0.5 µM TDZ was used. Shoot number, shoot height and leaf number per shoot increased with sub-culturing up to the second sub-culture period and then stayed the same (Fig. 3).

Liquid culture system improved the efficiency of *in vitro* propagation of three roseroot clones (Fig. 1D); shoot number was about 3-4 times more than those on gelled medium for all three clones (data not shown). Besides, less TDZ concentration (0.5 µM) is required in liquid culture compared to gelled medium (4 mM) for maximum number

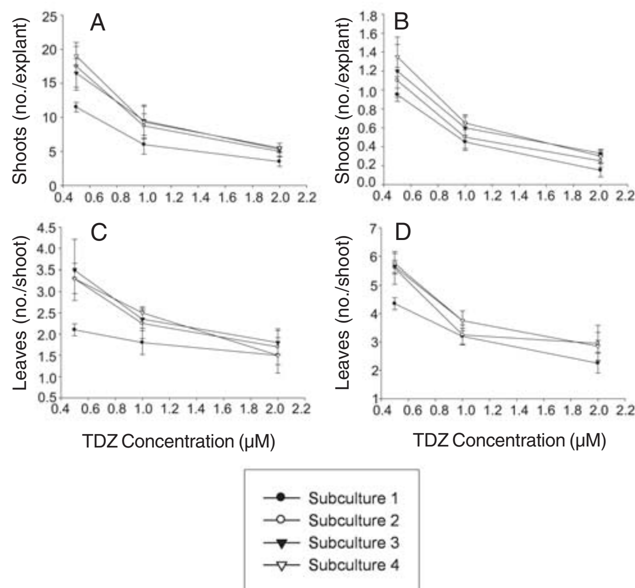

Fig. 3. The influence of varying concentrations of thidiazuron (TDZ) on shoot multiplication of roseroot clone 'RC1' grown over four sub-culture periods in a bioreactor vessel containing liquid medium for 8 wk: (**A**) shoots (no./explant), (**B**) shoot height (cm), (**C**) leaves (no./shoot), and (**D**) shoot vigour (scored on a scale from 1 to 8, with the poorest shoot being 1 and 8 the best). Vertical bars indicate ±SD.

of shoot production. However, hyperhydricity was observed in 20-30% of the shoots of all clones after 8-10 wk of culture in liquid BM with 1.0 μM TDZ (data not shown). On the other hand, hyperhydricity symptoms were almost absent among roseroot clones proliferated on gelled medium with low concentration of TDZ (1μM). It was possible to significantly improve the quality of shoots multiplied in liquid culture after transferring them onto gelled BM with 1-2 μM zeatin. Nornal shoots were obtained within 4 wk of transfer of hyperhydric shoots onto gelled medium (data not shown).

Shoots of all three clones rooted *in vitro* in the growth-regulator-free BM within 5-6 wk of culturing with a frequency of 90 to 95% in all three clones. Plantlets obtained *in vitro* (Fig. 1E) were adapted and transferred to soil with a survival rate of 85 to 90%. After acclimatization, plantlets have grown actively in the greenhouse with an apparent normal leaf and shoot morphology (Fig. 1F).

In conclusion, this report presents a protocol for roseroot micropropagation for the first time in a bioreactor system. Multiple shoot proliferation can be obtained by culturing in the liquid medium with 0.5 μM TDZ in a bioreactor system for 8 wk to scale-up shoot multiplication. Such TDZ-induced shoots can be rooted easily on PGR free medium and established in the greenhouse. The results from this investigation can be used for the conservation and large-scale propagation of roseroot.

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