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BRIEF COMMUNICATION

Micropropagation of *Karwinskia parvifolia* and the transfer of plants to *ex vitro* conditions

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Abstract

Zygotic embryos of *Karwinskia parvifolia*, isolated from seeds obtained from different regions of Mexico, were cultured on Woody Plant Medium (WPM) supplemented with 0.06 μM indole-3-acetic acid, 0.03 μM gibberellic acid, and 2 μM 6-benzylaminopurine. The growth of embryos and multiplication of shoots from stem segments were achieved. Rooting of excised shoots could be initiated on basal WPM medium with prolonged subculture period to 2 months, or on WPM medium supplemented with 10 μM 1-naphthaleneacetic acid. Multiplication capacity of shoots and rooting of *K. parvifolia* differed in dependence on the origin of explant material. The shoot multiplication was much lower than that of *Karwinskia humboldtiana*. The rooting depended on the origin of *K. parvifolia* seeds. The regenerated plants were successfully transferred to glasshouse.

Additional words: *in vitro* cultures, medicinal plants, *Rhamnaceae*.

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Abbreviations: BAP - 6-benzylaminopurine, GA_3 - gibberellic acid, IAA - indole-3-acetic acid, NAA - 1-naphthaleneacetic acid.

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Chemical and toxicological screening of nine species of the genus *Karwinskia*, which represent all of the species known in Mexico, showed the presence of dimeric anthracenonic compounds (Waksman *et al.* 1989). These metabolites are the source of the toxicity of these plants to mammals. One of them, T-514, shows antitumour effects on mammalian cells (Piñeyro López 1990), and specific activity on peroxisomes in yeast (Sepúlveda *et al.* 1992). The above characteristics form the basis of the current interest in these substances and in the genus (Waksman *et al.* 1989, Rivas *et al.* 1990). However, the content of T-514 is highly variable in different species, and also depends upon the way and time of collection. This was one of the reasons for experiments with the cultivation of *K. humboldtiana in vitro* (Lišková *et al.* 1994) which could avoid this problem. *K. humboldtiana* showed high regeneration capacity *in vitro* - multiplication of shoots (16.1 + 2.26 shoots per explant in the first year of culture and 22.6 ± 1.58 in the second year) and their rooting. The presence of T-514 in plants developed *in vitro* was ascertained (Argalášová 1996). Dark electrondense material and globular structures associated with tonoplast were present in primary calli initiated on root cultures (Lišková *et al.* 1994). Because of the production of these unidentified dark substances in vacuoles, the accumulation of some secondary metabolites in callus tissue may also be possible. Since *K. parvifolia* has been shown to have the highest content of the T-514 compound of all *Karwinskia* species (Waksman *et al.* 1989), it seemed relevant to produce *in vitro* cultures of this species and compare their regeneration capacity with those of *K. humboldtiana*.

The aim of this work was to establish optimum *in vitro* conditions for regeneration and micropropagation of *K. parvifolia* and to examine the viability of the resultant plants when transferred to glasshouse conditions. *K. parvifolia* is at present intensively studied species not only because of its highest content of T-514, but also for the most suitable composition of toxins, which reduces the price for the purification of T-514.

Seeds of *K. parvifolia* were obtained from immature (green) and mature (black) fruits collected from different regions of Mexico: Sauz, Batosori and Melchor Ocampo. Before use all seeds were stored at 4 °C for 1 month.

Imbibition and sterilization were carried out as described previously for *K. humboldtiana* (Lišková *et al.* 1994). Embryos were isolated from seeds with a lancet and post-sterilized in 0.5 % SAVO (commercial bleach - 20 % v/v; *Lachema*, Brno, Czech Republic) for 5 min and washed 3 times (for 5 min each) with sterile distilled water.

Isolated embryos were placed onto Woody Plant Medium - WPM (Lloyd and McCown 1981) supplemented with 0.06 μM IAA, 0.06 μM GA_3, and 2 μM BAP which was used as the basal medium for embryo development and shoot multiplication from stem segments excised from shoots developed *in vitro*. These shoots were rooted on the same medium with prolonged subcultivation period to 8 weeks, or on WPM medium supplemented with 10 μM NAA. All growth hormones were added to the media before autoclaving.

Isolated embryos were preincubated in dim light (4 μmol m^{-2} s^{-1}) at 28 °C for 48 - 72 h and then cultured under a 16-h photoperiod (irradiance of 45 - 60 μmol m^{-2} s^{-1}), temperature of 23 °C, and relative air humidity of 60 %, or directly in the latter conditions. Organ cultures were cultivated under the same conditions.

Rooted plants were subcultured into 250 cm^3 Erlenmeyer flasks with perlite supplemented with a solution of macroelements (Lloyd and McCown 1981), and cultivated for 2 months at 23 ± 1 °C, under shade. The plants were then subcultured in a mixture of soil with perlite (2:1), covered with glass beakers to keep the humidity high, and grown in the glasshouse with day and night temperatures of about 35 °C and 20 °C, respectively. The plants were hardened by gradually increasing the periods of exposure to ambient humidity from 1 to several hours. After 1 month the plants were transferred to free air.

All experiments were carried out three times, about 400 explants were used altogether. The results were expressed in percentages, and the mean ± standard errors (S.E.). The significance of differences in regeneration capacity of *K. parvifolia* and *K. humboldtiana,* as well as *K. parvifolia* from different regions was calculated by the Student's *t*-test.

Our attention is focused on the production of tissues, organs and whole plants *in vitro* which could serve as continual producers of secondary compounds. *K. humboldtiana* showed high regeneration capacity in cultures *in vitro* (Lišková *et al.* 1994). Recently *K. parvifolia* has attracted attention because of its high contents of T-514, therefore seeds of *K. parvifolia* from different regions of Mexico were collected to study the regeneration capacity of this species. The most successful results were obtained on the WPM medium under the same conditions as used for *K. humboldtiana* (Lišková *et al.* 1994). The vitality of embryos was substantially increased by preincubation in dim light for 48 - 72 h, which is known also for *K. humboldtiana* (Fig. 1). The vitality of the embryos varied between

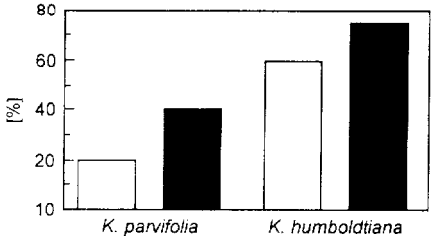

Fig. 1. Effect of preincubation (48 - 72 h at 28 °C in dim light) on the percentage of *K. humboldtiana* and *K. parvifolia* (independent on the origin of seeds) growing embryos after a period of 7 d in *in vitro* culture. *Empty columns* - embryos without preincubation, *full collumns* - preincubated embryos).

the two species, and distinctions were observed also in the differentiation ability. Multiplication of shoots of *K. parvifolia* depended on the origin of seeds (all data were highly significant with the exception of *K. parviflora* from Sauz and Batosori. The regeneration potential - multiplication of shoots and subsequent rooting - was 95 % in *K. humboldtiana* and 58 % or less in *K. parvifolia* (independent of the collection area). This might be a consequence of differences in the vitality of the isolated embryos.

Immature zygotic embryos isolated from seeds originating from the collection region Sauz produced mostly feeble plantlets with low multiplication of shoots (2.12 ± 0.24 per explant per subculture). The multiplication of shoots was mostly initiated from nodal areas of excised primary shoots developed *in vitro*. However, most shoots (89 %) produced very long firm roots (Fig. 2a) on basal WPM medium. No substantial differences in the morphology of roots were observed when the basal medium was enriched with 10 μM NAA. In contrast in *K. humboldtiana* (Lišková *et al.* 1994) the roots were thicker and covered with callus-like tissue. Compared to root cultures

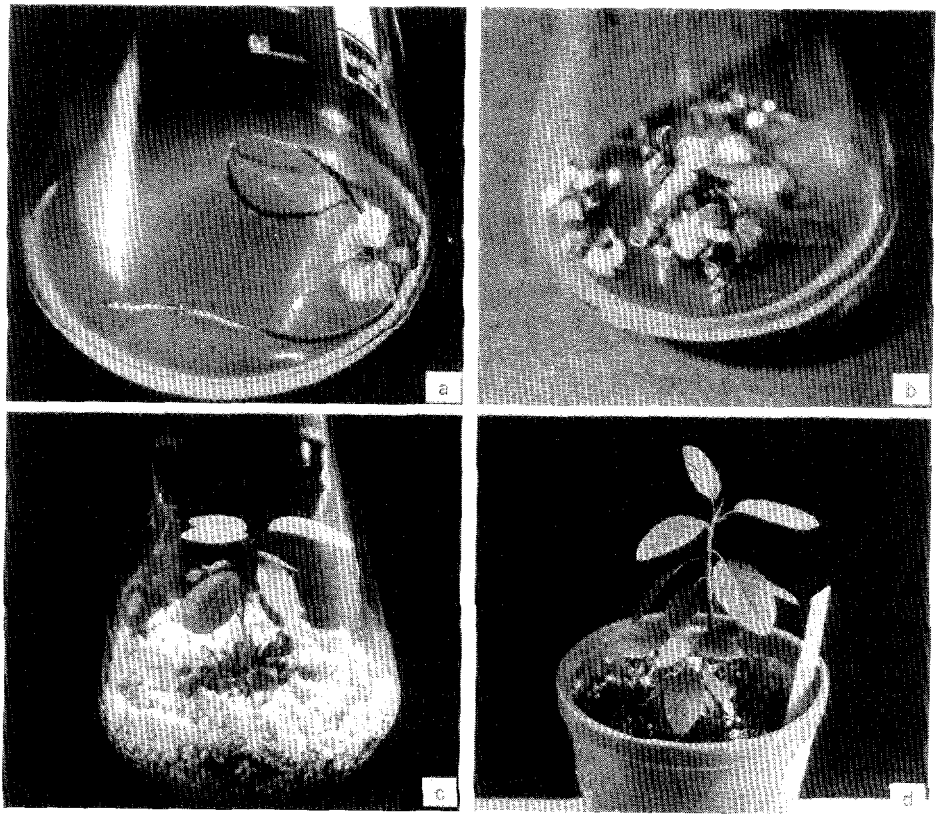

Fig. 2. Cultivation of *Karwinskia parvifolia in vitro* and its transfer to glasshouse conditions: *a*) growth of a plantlet with a long root on WPM medium, after 4 weeks of culture; seeds obtained from Sauz; *b*) multiplication of shoots, second subculture of excised stem segments - 8 weeks after initiation of shoot multiplication; seeds obtained from Batosori; *c*) transfer of rooted plants to perlite - 12 - 14 weeks after initiation of shoot multiplication; seeds obtained from Sauz; *d*) plant adaptation to glasshouse conditions - after about 19 - 20 weeks of the initiation of shoot multiplication; seeds obtained from Sauz.

of *K. humboldtiana*, initiated and grown successfully on modified Murashige-Skoog medium (Lišková *et al.* 1994), no root cultures of *K. parvifolia* were achieved. Dark pigmented hard calli were formed from roots that later became necrotic.

Immature embryos from the area Batosori formed very feeble roots. The multiplication of shoots on excised primary shoot explants was higher (5.28 ± 0.5 per explant per subculture) compared with the last one (Fig. 2b). However, the initiation of roots was successful only in about 1 % of cultures.

Embryos from the Melchor Ocampo area were isolated only from mature seeds. Under the above conditions these embryos did not form roots at all, only a dark hard callus appeared. The multiplication of shoots, induced already from hypocotyls, was very poor (0.8 ± 0.04 per explant per subculture). This low regeneration potential of the plant material obtained from this collection area might have been connected with the age of isolated zygotic embryos and their origin.

A seasonal effect in the regeneration potential, with a preference for spring was noted. Both long-term cultures of *K. parvifolia* and their transplantation to perlite was successful only from the material obtained from the Sauz (Fig. 2c). Subsequent transfers to a glasshouse were relatively easy, and the majority of plants (about 90 %) transferred to *ex vitro* conditions grew successfully (Fig. 2d). The plants were adapted to these new conditions by gradually increasing the periods with lower humidity, and after about 1 month they were grown in regular glasshouse conditions. This demonstrates the high adaptability of these plants which may be related to the severe semiarid conditions of their areas of origin.

The results obtained indicate considerable differences in the regeneration ability *in vitro* between the two *Karwinskia* species, as well as in the vitality of *K. parvifolia* embryos isolated from seeds of different origin, influenced probably also by local environmental conditions.

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