In vitro establishment and proliferation of red currant cultivars

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Abstract

Sedlák J., Paprštein F., 2012. ***In vitro* establishment and proliferation of red currant cultivars**. Hort. Sci. (Prague), *39*: 21–25.

The goal of this study was to investigate *in vitro* multiplication protocols for use with red currant cultivars grown in the Czech Republic. Cultivars Detvan, Vitan and Rotte Höllandische were successfully established *in vitro* using mercuric chloride in a concentration of 0.15% as a sterilization solution. The overall rate of contamination was 25.7%. Two proliferation media Murashige and Skoog medium (MS) and McCown woody plant medium (WPM) containing 1 or 2 mg/l of 6-benzylaminopurine (BAP) were tested. Initial explants produced new plants in the form of rosettes. Rosettes arose from the base of the initial explants in the form of adventitious bud formation. The shoot number was relatively low and varied between 1.0 and 2.1. Generally, the highest number was obtained for cultivar Rotte Holländische that produced 2.1 ± 0.1 new rosettes on MS medium containing lower concentration 1 mg/l BAP. In contrary, Vitan cv. had significantly lower shoot number ranging from 1.0 to 1.3. WPM medium with a lower concentration of mineral salts proved to be unsuitable for the multiplication of tested cultivars.

Keywords: *Ribes*; medium; explant; 6-benzylaminopurine; growth regulator

The red currant (*Ribes rubrum* L.) is a member of the genus *Ribes* in the gooseberry family *Grossulariaceae*, and is a popular bush fruit grown widely in the Czech Republic, especially in home gardens. Fruits are eaten raw or cooked into syrups, jams and jellies. In recent *in vitro* studies, red currant was identified as a good dietary source with potential antidiabetes and antihypertension functionality (da Silva Pinto et al. 2010). Concerning the current commercial currant growing, there were 1,002 ha of red and white currant and 345 ha of black currant plantations in the Czech Republic in 2009 (Buchtová 2009).

Commercially, it is almost entirely propagated by hardwood cuttings. This method, although generally successful, is slow and labor intensive. Success with rooting of cuttings also varies with the individual genotype, age of the stock plant and vegetation period (Hričovský 1985; Schubert et al. 2001). Micropropagation has the ability to quickly produce large numbers of shoots from a relatively small amount of initial plant material. It is especially effective in the case of new varieties, when clonal material is limited in supply. It also enables the maintenance of plant material in a pathogen-free environment and the development of sophisticated *in vitro* virus elimination techniques (*in vitro* thermotherapy and chemotherapy).

Data on *in vitro* currant culture are quite limited in comparison to other fruit woody species. Over the

Supported by the Ministry of Agriculture of the Czech Republic, Project No. QH91224.

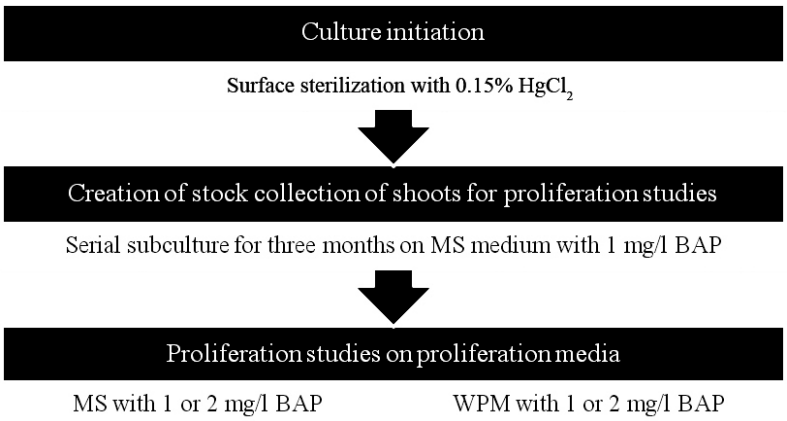

Fig. 1. Stages of micropropagation

last three decades, some reports indicated that *in vitro* culture propagation on various basal media using axillary bud proliferation and adventitious shoot regeneration were achieved with varying success for different species from the *Ribes* genus (Flegmann, Wainwright 1981; Wainwright, Flegmann 1986; Orlikowska et al. 1991; Arena, Pastur 1995; Benson et al. 1996; Karjalainen et al. 2001; Golis et al. 2002; Ruzic, Lazic 2006). However, reported number of newly formed shoots usually did not exceed 3–4 shoots per original explant. The response of individual genotypes also varied during the whole cycle of micropropagation (Leontiev-Orlov et al. 2004; Ruzic, Lazic 2006).

The work reported here was designed to investigate *in vitro* multiplication protocols for use with commercially important red currant cultivars grown in the Czech Republic as a part of the program for sanitation of initial plant material from viruses by *in vitro* thermotherapy and chemotherapy.

MATERIAL AND METHODS

The initial explants were actively growing shoot tips (5–10 mm in length) taken from shoots of red currant cultivars Detvan (154 shoot tips), Vitan (101 shoot tips) and Rotte Höllandische (130 shoot tips) sprouting in laboratory conditions. The donor dormant shoots were obtained in February from mature bushes growing in field collections of Research and Breeding Institute of Pomology (RBIP) Holovousy, Ltd., Holovousy, Czech Republic. The apical parts of shoots were prepared for sterilisation procedure by removing all expanded leaves. They were sterilised by a 1-min immersion in a fresh 0.15% $HgCl_2$ solution with several drops of wetting agent added (Tween-20). This was carried out under sterile conditions. All sterile manipulations were in a laminar flow hood. After thorough rinsing in sterile deionised and demineralised water, the explants were placed in 200 ml glass culture flasks (five shoots per flask), each with 35 ml of culture medium. The culture medium contained Murashige, Skoog medium (MS) salts and vitamins (Murashige, Skoog 1962) supplemented with 100 mg/l inositol, 2 mg/l glycine, 30 g/l sucrose, 1 mg/l 6-benzylaminopurine (BAP) and 7.0 g/l Difco Bacto agar. The medium was adjusted to pH 5.8 with NaOH prior to dispensing and autoclaving at 120°C at 100 kPa for 15 min. All shoot cultures were grown at 22 ± 1°C with a 16-h daily photoperiod. The irradiance was provided by Sylvania F18W (Sylvania, Erlangen, Germany) cool white fluorescent tubes positioned 30 cm above the level of cultures. If any exudation from the initial explants occurred, they were moved to fresh medium. At this time any malformed tissues were discarded. All shoot cultures were serially subcultured for three months on a MS medium supplemented with 1 mg/l 6-benzylaminopurine (BAP). This provided a stock collection of shoots for proliferation studies.

Two proliferation media MS and WPM (Woody Plant Medium) according to Lloyd and McCown (1981) containing 1 or 2 mg/l BAP were tested from the beginning of the culture. Proliferation rate was defined as the number of newly formed shoots (rosettes > 5 mm) per initial *in vitro* plant after four weeks of culture. Cultures were transferred to fresh medium once a month. At each subculture the explants were removed and dissected to determine the number of rosettes that had proliferated from the initial single *in vitro* plant. This sequence was repeated three times and a minimum of four culture flasks per cultivar was recorded on each occasion. The rosettes formation was recorded between the first and seventh subculture. Various stages of micropropagation are included in Fig. 1. Treatment means were compared with the standard error

(SE) of the mean. Analysis of variance (ANOVA) and Duncan's multiple range test were performed to analyze shoot number rates. For the three red currant cultivars, the effect of two agar solidified media (MS, WPM) and BAP growth regulator in two concentrations on proliferation rate, callus formation and shoot morphology is shown.

RESULTS AND DISCUSSION

Results of sterilization procedures and development of shoots from initial explants are stated in Table 1. Selected three genotypes of red currant were successfully established *in vitro* using mercuric chloride in a concentration of 0.15% as a sterilization solution. The rate of contamination for particular cultivars was 8.4% for Detvan, 31.5% for Rotte Holländische and 44.6% for Vitan. The overall rate of contamination was 25.7%. All contaminated explants were later discarded. *In vitro* culture was established from remaining actively growing uncontaminated explants. During and after our sterilization procedure, we did not observe browning of currant explants or cultivation medium caused by oxidation of phenolic substances from the cut surface of the explant, which is otherwise associated with woody species. Arena and Pastur (1995) obtained for greenhouse-grown *Ribes magellanicum* 70% survival and only 12% contamination after the disinfestation procedure of initial explants with NaOCl (1% active Cl). In our study, bacterial and fungal contaminations after sterilization of initial explants were more frequent, probably because the donor shoots were directly exposed to field microflora.

After 4 weeks in culture, ca. 90% initial explants survived and produced new plants in the form of rosettes with circular arrangement of well developed leaves, shortened internodes along a stem and a short, but well developed shoot tip. Rosettes appeared to arise from the base of the initial explants in the form of adventitious bud formation. In our experiments on both media, any physiological disorders or morphological abnormalities such as excessive callus formation or production of abnormally narrow leaves were not observed during *in vitro* shoot proliferation stage. Further dividing and subculturing the basal shoot mass did not cause tissue breakdown or exudation.

The number of newly formed shoots for particular cultivars is shown in Table 2. Values of multiplication rate were relatively low and varied between 1.0 and 2.1. Generally, the highest rate was obtained for cultivar Rotte Holländische that produced 2.1 ± 0.1 new rosettes on MS medium containing lower concentration 1 mg/l BAP. Similar number of newly formed shoots (2.0 ± 0.1) was obtained on the same medium with the same lower BAP concentration for Detvan cv. Across both media and BAP concentrations the rosette multiplication of Rotte Holländische cv. was the best. On the contrary, for cultivar Vitan, none of the two me-

Table 1. Surface sterilization with 0.15% $HgCl_2$

Cultivars (number of sterilized initial explants)	Explants contaminated		Established explants which developed shoots	
	number	(%)	number	(%)
Detvan (154)	13	8.4	141	91.6
Rotte Holländische (130)	41	31.5	89	68.5
Vitan (101)	45	44.6	56	55.4
Totally (385)	99	25.7	286	74.3

Table 2. Shoot number of red currant cultivars

Cultivar	MS medium		WPM medium	
	BAP 1 mg/l	BAP 2 mg/l	BAP 1 mg/l	BAP 2 mg/l
Detvan	2.0 ± 0.1^{a}	1.5 ± 0.1^{b}	1.0 ± 0.0^{c}	1.0 ± 0.1^{c}
Rotte Holländische	2.1 ± 0.1^{a}	2.0 ± 0.1^{a}	1.2 ± 0.1^{c}	1.0 ± 0.0^{c}
Vitan	1.3 ± 0.0^{bc}	1.0 ± 0.0^{c}	1.0 ± 0.0^{c}	1.0 ± 0.0^{c}

MS medium – Murashige and Skoog medium; WPM – McCown woody plant medium; BAP – 6-benzylaminopurine; $^{a–c}$mean values followed by the same letters are not significantly different at the 0.05 level of significance (Duncan's multiple range test)

dia and BAP concentrations promoted markedly *in vitro* shoot formation and the number of newly formed shoots was thus relatively low from 1.0 to 1.3. General appearance of the shoots was better at lower cytokinin content (1 mg/l).

The cultivars in this study differed in their multiplication and development potential. The effect of the genotype on the various aspects of the performance of tissue-cultured material was also reported in other studies on *Ribes* species (Wainwright, Flegmann 1985; Brennan et al. 1989; Orlikowska et al. 1991; Leontiev-Orlov et al. 2004; Ruzic, Lazic 2006). Our experiments confirmed that multiplication depends not only on the concentration of cytokinins in culture medium, but also on the response of individual genotype. The observed differences in multiplication among red currant cultivars in this study under the influence of an exogenous BAP could result from the genetic control of different auxin and cytokinin metabolisms of plant tissue. The studies of Brennan et al. (1990) on the *in vitro* culture of a range of *Ribes* cultivars, breeding lines and species showed clear differences in genotypic multiplication rates and cultural behaviour which suggest a link between multiplication rate and genetic composition related to geographical origin.

The lowest shoot number (1.0–1.2) was noted for all selected cultivars on WPM medium. This medium with a lower concentration of mineral salts proved to be unsuitable for the multiplication of tested cultivars. Explants of Rotte Holländische and Detvan cvs on WPM medium produced significantly lower shoot numbers at both BAP concentrations in comparison with MS medium. *In vitro* plants on WPM medium were of poor quality and developed symptoms of yellowing or chlorosis of leaves in later subcultures.

It was possible to maintain actively growing *in vitro* cultures of all used red currant cultivars on both tested media only for a limited number of subcultures (usually, 7–9 subcultures; total culture period about 9 months), after which they either ceased to proliferate and multiply or eventually turned yellow-brown and subsequently died out. There was no obvious reason for this decline in proliferation and multiplication. Similar phenomenon of not sustained rate of proliferation was described by Wainwright and Flegmann (1985) in their multiplication experiments with *Ribes uva-crispa* L. They described that the proliferation rate of two *R. uva-crispa* cultivars fell since the fourth subculture on MS medium with 1μM/l BAP and they questioned the sustainability of further subculturing beyond 170 days from culture initiation. In contrary Brennan et al. (1989), Toshkov (2004) and Ruzic and Lazic (2006) found that a BAP level of 0.5–2 mg/l in modified MS medium proved satisfactory for shoot induction and reproducible and sustainable development of a range of diverse *Ribes nigrum* genotypes.

From our studies, the red currant was the most difficult fruit species to propagate. The procedure was shown possible, but the levels of multiplication were low. It is sufficient for *in vitro* culture establishment, short-term maintenance and *in vitro* thermotherapy. But such a low shoot number is not satisfactory for larger *in vitro* shoot production in a commercial scale.

In near future, the use of micropropagation for *Ribes* species is unlikely to be widely used in commercial production, as there are already relatively slow but inexpensive and reliable traditional nursery techniques. *In vitro* propagation, which can be carried during the dormant season, could provide an alternative means to produce disease-free stock material. *In vitro* propagation techniques are also general prerequisites to exploiting somaclonal variation and induced mutations, and for the development of transgenic plants.

CONCLUSSION

The use of mercuric chloride in a concentration of 0.15% as a sterilization solution proved to be an applicable treatment method to disinfect the starting plant material of selected red currant cultivars. It was found under the conditions employed that MS medium and BAP level of 1 mg/l is satisfactory for *in vitro* culture establishment and short-term maintenance of tested red currant cultivars, but not for sustainable development and multiplication in larger scale. More research is needed to develop a protocol suitable for commercial micropropagation. Other methods will be investigated, for example multiplication system according to Ruzic and Lazic (2006), which uses nodal sections taken from elongating *in vitro* shoots grown on media supplemented with low concentrations of indole-3-butyric acid (IBA) and giberellic acid (GA_3).

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Received for publication February 25, 2011
Accepted after corrections July 11, 2011

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