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ASPECTS REGARDING THE *IN VITRO* MULTIPLICATION OF THE *RUBUS HIRTUS* L. SPECIES

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***Key words**: Rubus, in vitro, caulogenesis, rhizogenesis, micropropagation*

INTRODUCTION

The genus *Rubus* is morphologically and genetically diversified, comprising approximately 237 species. Some of them have been valorised since ancient times, which is proven by writings attributed to ESCHYLUS and HIPPOCRATES (500 B.C.) and to botanist DISCORIDES (65 B.C.), in which the blackberry is praised for its benefits in different medical treatments. In broadleaf and coniferous forests of Romania, the most common variety is the creeping blackberry (*Rubus hirtus* L.), a frost-hardy species, adapted to the cold and humid Boreal climate. Its fruit are dark black-blue and are very sweet when ripe. At European level, blackberries became the fourth freshly consumed fruit after strawberries, blueberries and raspberries. They are also used in different ready-cooked products: candies, pastry and yoghurts. The fruit are used in the pharmaceutical and cosmetics industry, too. In their chemical composition were identified sugars, organic acids, minerals and vitamins. The high level of antioxidants determined an increased interest in these fruit in most European countries. However, the current yield of forest fruits is very low, a situation generated by the shrinking of wooded surfaces and inferior genotype quality.

The traditional propagation of blackberry species is achieved by marcotting or by cuttings, their success depending on genotype and seasonal conditions. Unfortunately, a lot of superior genotypes are difficult to root and the process requires a long period of time. An alternative to traditional propagation is *in vitro* culture, which allows obtaining a larger number of genetically identical plants, bearing fruit of better quality, within a shorter period of time. The first report on *in vitro* propagation in a *Rubus* species was achieved in 1978 by BROOME and ZIMMERMAN for thornless blackberry (*Rubus fruticosus*) and by HARPER (1978). Reference materials reported other attempts at *in vitro* culture for some blackberry species (BOBROWSKI, MELLO-FARIAS, PETERS, 1996), (NAJAF-ABADI, HAMIDOGHLI, 2009), (CLAPA D., FIRA A., PLOPA C., 2011) which used a Murashige & Skoog basal medium with additional concentrations of BAP, IBA, NAA and GA3. For the *Rubus pubescens* species, the initiation phase was conducted on Murashige & Skoog basal medium (1962), supplemented with BAP and IBA, whereas the propagation was achieved on a basal medium supplemented with Zeatin concentrations (DEBNATH, 2004). It has been reported that regeneration of adventitious shoots in blackberry has been done from different types of explants, i.e. whole leaves (SWARTZ et al. 1990; AMBROZˇIC´ TURK et al.1994; GUPTA and MAHALAXMI 2009), leaf sections and petioles (GRAHAM et al. 1997; MEZZETTI et al. 1997; MENG et al.2004), excised cotyledons (FIOLA et al. 1990) – cited by VUJOVIC´, 2010.

Considering specialists' interest in valorising some blackberry plantations, our research investigated the *in vitro* behaviour of some explants of indigenous *Rubus hirtus* L., to the purpose of identifying some rapid micropropagation technologies and the possibility of obtaining some new somaclones via callus.

MATERIAL AND METHODS

In order to initiate the *in vitro* culture, young blackberry shoots were used from spontaneous flora (mixed forest, altitude of approx. 500 - 550m) near Moineşti, in Bacau County. The biological material was washed and kept five days under laboratory conditions (22.5 ° C and 9:00 photoperiod).

The explants used were nodes and shoot apexes, of small sizes, so as to reduce the risk of infections. The explants collected were repeatedly washed under running tap water. The sterilization of the plant material was carried out with mercuric chloride solution 1 ‰ - 10 minutes and solution of Chloramine T 5 % - 20 minutes in a tissue culture hood. To remove the sterilizing solution, the explants were rinsed three times with sterile distilled water. To test morphogenetic reaction, explants were then inoculated on several nutritive variants of basal mediums including *Murashige - Skoog* (MS, 1962) and *Woody Plant Medium* (WPM, Lloyd and Mc Cowne, 1980), supplemented with different combinations and concentrations of growth

regulators. Table 1 presents the nutrient variants that induced a positive morphogenetic reaction.

The inoculated pots were incubated in a growth chamber at a temperature of 22.5 °C, 16 hours photoperiod and 2,500 lux intensity of light.

Table 1. Nutritive variants on which blackberry explants reacted

Crt. No.	Variant	Basal medium	Growth regulators
1	BB_1	MS	1 mg/l BAP + 0.5 mg/l IBA
2	BA_1	MS	1 mg/l BAP + 0.5 mg/l IAA
3	KA	MS	1 mg/l BAP + 0.5 mg/l IAA
4	BG_3	MS	1 mg/l BAP + 0.3 mg/l GA3
5	BK	MS	1 mg/l BAP + 1 mg/l Kin
6	WPM_1	WPM	2 mg/l BAP + 1 mg/l IBA + 0.5 mg/l GA3
7	WPM NAA 1,5	WPM	1,5 mg/l NAA
8	A_6	WPM	3 mg/l Zeatin + 0.5 mg/l IBA
9	A_8	WPM	2 mg/l Zeatin + 1 mg/l BAP
10	A_9	WPM	2 mg/l BAP + 1 mg/l Kin
11	A_{11}	WPM	1 mg/l BAP + 3 mg/l Kin

The first reactions of the inoculated explants were registered at approximately 2 weeks after inoculation.

Three repetitions of inoculations were performed on the same nutrient variant.

The callus obtained on certain hormonal variants was transferred to another nutrient medium which induced the regeneration of shoots (caulogenesis).

The shoots thus obtained were transferred to the rooting medium – WPM NAA1,5.

The accommodation of the vitroplants to the *ex vitro* medium was achieved through their transfer in hydroponic culture system. The acclimatisation was achieved in about two weeks, after which the plantlets were transferred to pots with sterile soil mixed with perlite, (Figure 11, 12).

RESULTS AND DISCUSSIONS

The initiation of the *in vitro* culture has shown some disadvantages in that the sterilization of biological material has presented some problems. Several stages of the sterilization process were conducted as we experienced great loss of plant material through the occurrence of infections. In the case of apex explants, there was a lower frequency of infections, due to their size, compared to node explants, and due to the fact that they passed in the laboratory through 5 days of 'quarantine'. To save the vegetal material in the contaminated pots, we appealed to the transfer of explants to new environments, accompanied by a new sterilization with a T5% chloramine solution.

Regarding the response in culture, the apex and node explants showed better caulogenetic response on most nutritive alternatives tested. The callus appearance was indicated, but with a low frequency and only on two hormonal combinations tested.

1 mg/l of BAP used on the culture medium was reported as being sufficient to favour the proliferation of shoots. Other authors observed the same situation at other species of the genus *Rubus* (WU, MILLER, HALL, & MOONEY, 2009). In our experiments, there were tested different concentrations of BAP, both simple and combined with other growth regulators.

The shoots on the BB_1 variant (1 mg/l BAP + 0.5 mg/l IBA mg/l) initially registered significant growth, then they had a slower growth. The leaves were larger and normally developed (Figure 1). The shoots transplanted on the same variant generated other shoots, their number being of 1-2/explants.

BA_1 variant (1 mg/l BAP + 0.5 mg/l IAA mg/l) had a similar influence on shoot development compared to BB_1 variant. They presented lent growth, while the leaves were also big and well-developed (Figure 2). Shoot maintenance on BA_1 variant, as well as on BB_1 for more than 6 weeks determined the appearance of chlorophyll deficiencies. Upon closer analysis of the shoots on the BA_1 variant, we found that they were characterized by a better start. The presence of the IAA phytohormone (0.5 mg/l) in the medium compared to IBA (0.5 mg/l), determined a better development of the shoots.

On BG3 variant (1 mg / l BAP + 0.3 mg / l GA3 mg / l), for *R. hirtus* species, there registered an increase in shoot height and the absence of chlorophyll deficiencies, even at 8 weeks of age. It seems that the use of GA_3 growth regulators was more advantageous than the use of IBA and IAA plant hormones in combination with BAP (Figure 3). The same combination of plant hormones (BAP and GA3) was tested by MARTINUSSEN, NILSEN SVENSON, JUNTTILA, and RAPP, (2004) for the species *Rubus chamaemorus*, but the morphogenetic reaction consisted of obtaining a reduced number of shoots per explant.A spectacular proliferation of shoots was observed on BK variant (1 mg/l BAP + 1 mg/l Kin mg/l) (Figure 4). The combination of the two Cytokinins induced the development of a much larger number of shoots compared to the previously discussed variants. The stems presented smaller shoots and leaves, but proportional to each other. After about 6 weeks of culture, the leaves of the shoots started presenting chlorophyll deficiencies, and then necroses. We consider that this phenomenon may be due to the abundance of the plant material in

the culture pot. On this variant, the proliferation of new shoots occurred even on the nutritive medium, an aspect which was not noticed on other hormone variant tested.

On the variant of the MS medium supplemented with 1 mg/l KIN and 0.5 mg/l IAA, the morphogenetic reaction of the explants consisted in the formation of both shoots and callus. The weakly proliferative, cream-coloured callus appeared at the base of the explants and at the level of basal leaves.

On both the callus and the stem, short white roots were formed. The shoots generated from the node and apex explants showed slow growth, but the leaves were well-developed. After approx. 4-5 weeks since their cultivation, the leaves presented chlorophyll deficiencies.

Caulogenesis represented the main morphogenetic reaction on the hormonal and environmental variants of WPM medium, and compared with the MS medium, rhizogenesis was clearly indicated.

WPM1 variant (2 mg/l BAP + 1 mg/l IBA + 0.5 mg/l GA3 mg/l) also registered a weak caulogenesis compared with all the variants tested on the MS medium. The shoots were weak and their sizes were similar to the ones obtained on the BK variant. Another noticeable aspect is the fact that the majority of shoot leaves presented chlorophyll deficiencies, some being albinotic (Figure 9). On this medium formula, there were obtained regenerants, as they degenerated in time. At a low rate of proliferation, there formed a greenish non-regenerative callus in the nutrient medium at the base of the shoots.

The interaction of the Zeatin and IBA phyto-hormones (A6 variant) determined the formation of new shoots from both node and apex explants (Figure 5). Their growth was good, the stem having sizes proportionate to the leaves. The phenomenon of leaf depigmentation was not registered. On this nutritive variant, at the base of some shoots, there formed long roots with a few secondary ramifications.

A8 variant (2 mg/l Zeatin + 1 mg/l BAP mg/l) was also a phytohormonal combination favourable to caulogenesis (Figure 8). There are a large number of shoots obtained as compared with the other variants tested; their frequency is close to that identified in the BK variant. In contrast, the shoots did not proliferate in the nutrient medium and the size of the shoots was significantly smaller compared with the other variants tested.

On WPM medium supplemented with 2 mg/l BAP and 1 mg/l Kin mg/l, (A9) the caulogenesis frequency was lower than the previous version and had larger shoots. This formula hormone also favoured the accumulation of high concentrations of anthocyanins in leaves (Figure 7). The survival of shoots is a problem, because their growth ceased and they degenerated in a short interval.

On A11 variant (WPM supplemented with 1 mg/l BAP + 3 mg/l Kin mg/l), the shoots degenerate shortly, having the same characteristics as the ones obtained on the A9 variant. The differences in the concentrations of the two phyto-hormones used in the two variants (2 mg/l BAP + 1 mg/l Kin in A9 variant and 1 mg/l BAP + 3 mg/l Kin in A11 variant) are not identifiable in the morphogenetic reaction (Figure 8).

CONCLUSIONS

The initiation of the *in vitro* culture in the *Rubus hirtus* species was difficult because of the inefficiency of the process of sterilisation of the vegetal material initially used.

The main morphogenetic reaction observed on the tested variants was generated on shoots, followed by rhizogenesis and, to a lesser extent, by caulogenesis.

The most indicated variants for caulogenesis induction are the ones of BK (1mg/l BAP + 1 mg/l Kin). For the Woody Plant basal medium, the best hormonal variant for obtaining shoots was A8 (2 mg/l Zeatin + 1 mg/l BAP mg/l), followed by A6 (3 mg/l Zeatin + 0.5 mg/l IBA).

Some nutrient media that we tested induced caulogenesis (on WPM1 and KA variants), but with low and non-regenerative proliferation.

From our observations and the ones registered in literature for the species belonging to *Rubus* genus, the BAP used alone or in combination with other plant hormones induces new regenerants in the *in vitro* culture.

The best results for shoot rooting were obtained on the WPM basal medium supplemented with 1.5 mg / l NAA.

Regarding the accommodation of in vitro-produced plants and their transplanting into the soil, no reported difficulties were reported.

ABSTRACT

The blackberry perennial shrub belongs to the *Rosaceae* family. It is among the oldest medicinal plants with Eastern origins, the proof of its medicinal use dates from Hippocrates times, the 4th century B.C. Its berries are appreciated for their taste and mostly for their high amounts of vitamins (A and C); the leaves have a medicinal utility, due to their disinfectant, healing, antihemorrhagic properties, and for hormonal regulation as well.

Due to its economic importance, the purpose of our research was to highlight the *in vitro* reaction of this species by observations on the morphogenetic response of a series of explants on various nutritive variants. At the same time, we aimed to identify a profitable technology to micropropagate blackberry.

In view of initiating the *in vitro* cultures, uninodal and apical explants were used, harvested from young shrubs, disinfested with $HgCl_2$ 1‰ and chloramine-T 5%. After the removal of disinfesting

solutions (by rinsing in distilled water), the explants were inoculated on various medium variants of basal MS (Murashige –Skoog,1962) and WPM (Woody Plant, Lloyd and Mc Cown, 1980) medium, enriched with several combinations and amounts of growth regulators.

Our observations led to the following conclusions: a proper morphogenetic response was noticed for the explants inoculated on the medium variants of MS enriched with 1mg/l BAP(benzylaminopurine) + 1 mg/l Kinetin, and WPM enriched with 2 mg/l Zeatin + 1 mg/l BAP; 3 mg/l Zeatin + 0.5 mg/l IBA (indole-3-butyric acid) - in order to micropropagate the shoots; the presence of ANA (naphtyl-acetic acid – 1.5 mg/l) within the WPM medium induced the development of a strong radicular system. The acclimatisation of the vitroplants to the *ex vitro* environment encountered no problems, and took place in a hydroponic system. About 2 weeks after the acclimatisation, the new plants were transfered into soil pots.

Fig. 1. Caulogenesis on BB1 variant

Fig. 2. Caulogenesis on BA1 variant

Fig. 3. Aspect of the shoots on BG3 variant

Fig. 4. Proliferation of shoots on BK medium

Fig. 5. Caulogenesis and Rhizogenesis on A6 variant

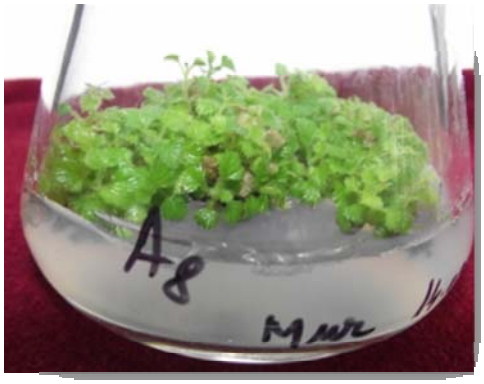

Fig. 6. Caulogenesis on A8 variant

Fig. 7. Shoots with anthocyanins in leaves on A9 variant

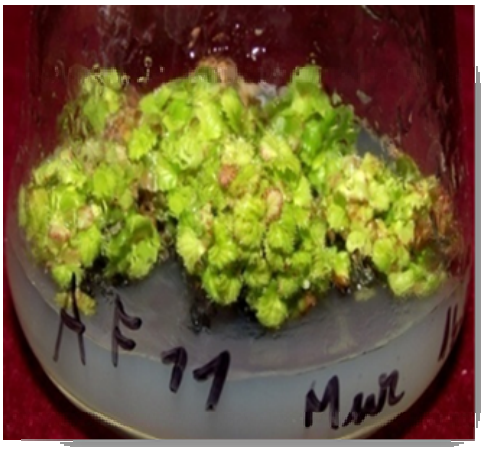

Fig. 8. Caulogenesis on A 11 variant

Fig. 9. Albinotic Shoots on WPM1 variant

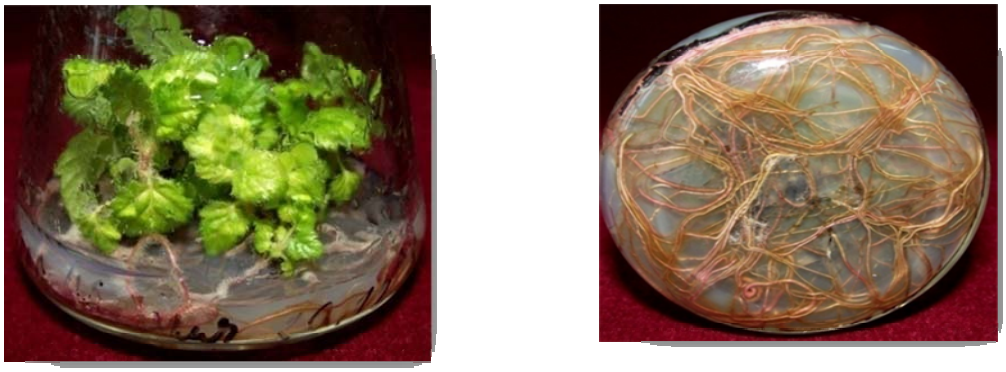

Fig. 10. Development of a strong radicular system and shoots on WPM 1,5 NAA variant

Fig. 11. The aspect of vitroplants

Fig. 12. Vitroplants in pots with sterile soil mixed with perlite

REFERENCES

1. BOBROWSKI V., MELLO-FARIAS P. C., PETERS J. A., 1996 - Micropropagation of blackberries (Rubus sp.) cultivars. Bras. de Agrociencia , 2 (1), 17-20;
2. CLAPA D., FIRA A., PLOPA C., 2011 - The micropropagation of some thornless blackberry cultivars. Scientific papers, R.I.F.G. Pitesti, Vol. XXVII;
3. CLARK J. R., FINN C. E., 2011 - Blackberry Breeding and Genetics. Fruit, Vegetable and Cereal Science and Biotechnology , 5 (1), 27-41;
4. DEBNATH S. C. , 2004 - Clonal Propagation of Dwarf Raspberry (Rubus pubescens Raf.) through in vitro Axillary Shoot Proliferation. Plant Growth Regulation, 43 (2), 179-186;
5. LIU C., CALLOW P., ROWLAND L. J., HANCOCK J. F., SONG G.-Q., 2010 - Adventitious shoot regeneration from leaf explants of southern highbush blueberry cultivars. Plant Cell, Tissue and Organ Culture (PCTOC) , 103 (1), 137;
6. MARTINUSSEN I., NILSEN G., SVENSON L., JUNTTILA O., RAPP K., 2004 - In vitro Propagation of Cloudberry (Rubus chamaemorus). *Plant Cell, Tissue and Organ Culture , 78* (1), 43-49;
7. NAJAF-ABADI J. A., HAMIDOGHLI Y. 2009 - Micropropaga□ion of thornless trailing blackberry (*Rubussp*.) by axillary bud explants. Australian Journal of Crop Science, 3 (4), 191-194;
8. WU J.-H., MILLER S.A., HALL H. K., MOONEY P. A., 2009 - Factors affecting the efficiency of micropropagation from lateral buds and shoot tips of Rubus . Plant Cell, Tissue and Organ Culture (PCTOC) , 99 (1), 17-25;
9. VUJOVIC T., RUJOVIC D., CEROVIC R., MOMIROVIC S. G., 2010 - Adventitious regeneration in blackberry (*Rubus fruticosus* L.) and assessment of genetic stability in regenerants. Plant Growth Regulation, 61 (3), 265-275.

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