III. ÎNMULŢIRE, VIROLOGIE ŞI CULTURI DE ŢESUTURI
PROPAGATION, VIROLOGY AND TISSUE CULTURE

ÎNMULŢIREA SOIURILOR NOI DE MUR PENTRU PRODUCEREA MATERIALULUI DE ÎNMULŢIRE CERTIFICAT
PROPAGATION OF NEW BLACKBERRY CULTIVARS FOR PRODUCING CERTIFIED PROPAGATION MATERIAL

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Abstract

The purpose of this research was to study the behaviour of two new Romanian thorn blackberry cultivars DAR 24 and DAR 8 in process of micropropagation, compared with Darrow cultivar usually commercially micropropagated in our laboratory. Due to several drawbacks of the conventional propagation of *Rubus*, the efficiency of micropropagation was tested in order to obtain high quality planting material and to introduce rapidly these new cultivars in a certification program. Thorn blackberry cultivars DAR 8 and DAR 24 with resistance to winter colds was successfully micropropagated. Blackberry plants were found without virus infection after biological and ELISA tests. Axillary buds from the branches in full growth were used as the initial explants. After four weeks of growth, aseptic cultures was established on MS basal mineral salts, LS vitamins with 0.3 mg/l BAP, 0.1mg/l GA_3 and 0.001mg/l NAA. The rate of successfully established cultures was on average 65.11%. Good proliferation of the regenerated shoots was obtained on the same medium composition used for initiation phase, whereas medium MS with mineral salts reduced to ½ and LS vitamins with 0.1 mg/l IBA and 0.1 mg/l GA_3 was used in the rooting phase. Dar 24 and Dar 8 cultivars responded by good rates of micropropagation on medium culture B as compared to control Darrow cultivar, even if the obtained shoots length was lower than on medium A. The statistical analysis reveled that the highest MR (20.66 plantlets/explant) was obtained for Dar 24. In this case the length of shoots was 1.92 cm. The highest rooting percentages (over 85%) were obtained with shoots multiplied on medium B. High quality of rooted plants induced a high percentage of acclimatization of cultivar Dar 8, 86.36% under mist system in green house whereas the percentage of acclimatization of cultivar Dar 24 was lower, 51.85%.

Cuvinte cheie: *Rubus fruticosus*, mur cu spini, muguri axilari, micropropagare
Keywords: *Rubus fruticosus*, blackberry thorns, axillary bud, micropropagation

1. Introduction

Blackberry belongs to the *Rubus* (Tourn) L. genus and *Eubatus* subgenus. Methods of asexual *Rubus* propagation include tip layering, various types of cuttings, and removal of sucker plants. Low numbers of propagules can be obtained, by traditional techniques. Broome and Zimmerman, 1978 mark out some disadvantages of traditional propagation techniques: tip layering propagation requires a sizeable planting for the layer bed, few tips are available per plant, and weed control among the layers is a problem. Propagation by hardwood stem is not always satisfactory and softwood cuttings root readily, but require considerably more care for successful plant production. Development of *in vitro* tissue culture techniques has demonstrated its application in rapid clonal propagation, regeneration and multiplication of selected genotypes. Application of in vitro propagation has been recorded in great number of blackberry cultivars (Donnelly et al., 1980; Ruzic and Lazic, 2006). At national level blackberry micropropagation has been investigated especially with thornless cultivars (Coman, 1981; Fira et al., 2009, 2010) and to improve *in vitro* propagation biotechnology (Neculae and Teodorescu, 1995). At RIFG Pitesti, micropropagation of more than 20 blackberry cultivars was studied with good results. Propagation systems approved by actual legislation regarding production of fruit tree planting material include also micropropagation technique. Therefore, the objective of this research was to study the behaviour of two new Romanian blackberry thorns cultivars DAR 8 and DAR 24 in process of micropropagation, compared with Darrow cultivar usually commercially micropropagated in our laboratory. The aim is to introduce these new cultivars in a certification program to obtain virus – free material and a rapid dissemination in culture. DAR 8 and DAR 24 cultivars with resistance to winter colds have medium length and density thorns and were created for fresh consumption and processing. Lately, they have been recommended in culture with Darrow variety for hardiness and productivity.

2. Material and methods

Cultures were initiated with biological material from selected and authentic plants tested for specific viruses (*Strawberry mild yellow edge* – SMYEV, *Arabis mosaic* – ArMV, *Strawberry latent ringspot* – *SLRSV, Raspberry ringspot* – RpRSV, *Tomato black ringspot* – TBRV, *Cucumber mosaic* – CMV, *Apple mosaic* – ApMV, *Cherry leaf spot* – CLRV, *Raspberry bushy dwarf* – RBDV) by double antibody sandwich DAS - ELISA according to Clark and Adams (1977) using commercially available kit supplied by Bioreba and following manufacturer`s protocol, and by biological tests. Regents from Bioreba are optimized for use in DAS –ELISA using certified Nunc-Immuno Plates Maxi Sorp F 96 and operating with a working volume of 200µL per well. Optical densities (OD) were recorded at 405 nm and readings were done with a Bio-Rad plate reader PR 2100. To detect the viruses transmitted by aphids (*Black raspberry necrosis* – BRNV, *Raspberry leaf mottle* – RLMV, *Raspberry leaf spot* – RLSV şi *Rubus yellow net* – RYNV) the grafting technique `bottle grafting` on indicator *Rubus occidentalis* `Munger, was employed.

In vitro cultures were established by use of axillary buds as initial explants. The plant material was washed with running water and after dezinfection was rinsed with sterilized deionized water. The shoots were cut in small segments and surface sterilized in 70° ethanol for 10 minutes followed by 20 min. in sodium hypochlorite (2% available chlorine). The buds were placed in tubes containing 10 ml of culture initiation medium containing major and minor salts as in Murashige and Skoog (1962) (MS) and Linsmaier - Skoog (1965) (LS) vitamins, supplemented with 0,3 mg/l N6 - benzilaminopurine (BAP), 0.1 mg/l gibberellic acid (GA_3) and 0,001 naphtylacetic acid (NAA). Explant regeneration (%) was recorded after 4 weeks of culture and the new shoots were transferred to proliferation medium.

We prepared two multiplication variants: medium A containing MS, Lee – Fossard (1977) and LS supplemented with 0,5 mg/l BAP, 0,5 mg/l GA_3 and 10 mg/l ascorbic acid, and medium B containing containing MS major and minor salts and LS vitamins, supplemented with 0,3 mg/l BAP, 0.1 mg/l GA_3 and 0,001 NAA. The multiplication rate and the lenght of obtained shoots was recorded after 3 month of culture. In the rooting experiment were used as initial explants nonrooted shoots separate from multiplication medium A and B. The mineral basic medium used was MS, reduced at 1/2 mineral salts, and LS vitamins, with 0,1 mg/l 3 – indolylbutiric acid (IBA) and 0.1 mg/l GA_3. Percent rooting was recorded after 4 - 6 weeks taken in account the provenience of material. Shoots with at least one root was considered rooted. All media have content NaFeEDTA and sucrose and were solidified with 7 g/l plant agar. For multiplicatin and rooting studies shoots were placed in Erlenmayer flasks containing 25 ml of culture medium. Plant tissue culture media are sterilized by autoclaving at 121°C and 1.5 bar (150kPa). Sterilization takes 20 min. *In vitro* cultures were maintained in the growth chamber at 22-24°C, and photoperiod of 16/8 h of light/darkness. Light intensity provided by white fluorescent lamps ranged between 32-40 µmol m^{-2}s^{-1}. For acclimatization study rooted plantlets were transferred to a perlite substrate in the green house.

Data were taken from three replicate experiments and analysed statistically by Duncan`s multiple range test at $P \leq 0.05$. All studies were conducted once.

3. Results and discussions

Virus detection

Evaluation of biological indicators used for viruses diagnosis, showed absence of specific symptomatology of viruses BRNV, RLMV, RLSV, RYNV and RYSV. Serological DAS-ELISA test also revealed, that plant material from all three cultivars was virus free (Table 1).

Establishment of in vitro culture and shoot multiplication

The results of explants sterilization and development of shoots from initial explants are recorded in Fig. 1. The sterilization procedures were successful and bacterial and fungal contaminants were infrequent. Of the total explants of three cultivars taken, only 13.05% on average were contaminated. After four weeks of growth on initiation medium the rate of successfully established cultures was 63.77%. There were differences concerning the behaviours of the *in vitro* cultures of both newly blackberry cultivars. Higher values of explant regeneration were found in Dar 24 cultivar, 71.92% followed by Dar 8 cultivar with 60.36%.

In the first stage of micropropagation process all three blackberry cvs. had a good reaction on medium A. Hormonal balance consists of 0.5 mg/l BAP, 0.5 mg/l GA_3 determined not only a good MR but also good shoots growth. The average of MR on medium A was between 10.3 shoots/explant at Darrow cv. and 15.33 shoots/explant at Dar 24 cv.. After three months of culture, higher values of MR were found on culture conditions of medium B with reduced concentration of growth regulators (0.3 mg/l BAP, 0.1 mg/l GA_3) and supplemented with 0.001 mg/l NAA. MR recorded was between 12 shoots/explant and 20.66 shoots/explant, (Fig. 2) but shoots had lower heights (Fig. 3). Reduction of GA_3 concentration influenced the shoots growth and stimulated the cytokinin action. Cytokinins like BAP in our experiment are very effective in promoting direct or indirect shoot initiation. It was known that GA_3 can synergistically act with auxins (Stowe, 1957), so that the presence of 0.001 mg/l NAA in medium favoured *in vitro*

multiplication. The statistical analysis revealed that as compared to the Darrow cv., Dar 8 and Dar 24 cvs., responded by a better MR after three months of culture. Statistical differences are found in the variety Dar 24 which has a multiplication rate significantly higher than the other two varieties. A parameter for the quality evaluation of plantlets obtained from multiplication phase was shoot length. So, the average length of shoots, irrespective of basic medium culture or combination of growth regulators, varied between 1.65 cm (Darrow) and 1.98 cm (Dar 24), Fig.3. Our data showed that on medium B apical dominance was reduced and the MR was higher but the obtained shoots length was lower than on medium A.

Rooting and acclimatization

The experimental results regarding rooting of blackberry microshoots indicated that the MS reduced at 1/2 mineral salts, and LS vitamins, with 0. 1 mg/l IBA and 0.1 mg/l GA_3 was very good medium for rooting all three blackberry cvs. Higher values of rooting percentage (>80%) were recorded, irrespective of shoots multiplication origin, medium A or B. High rooting percentages, ranging from 86.3% to 95.3% were obtained with shoots multiply on the B medium. Also, in the process of rooting there was statistical difference among cultivars. In this phase rooting percentages with shoots from multiplication medium B were higher in Dar 8 cultivar (95.3%) recording a significant difference from varieties Dar 24 (86.3%) and Darrow (90%), Fig. 4.

At the acclimatization stage about 58.32% of rooted microcuttings survived in greenhouse under mist conditions. As shown by Duncan`s multiple range test, Dar 8 cultivar had the best behavior with 86.36% acclimatization, compared with cultivar Dar 24 with only 51.85% acclimatization and Darrow with 36.76% acclimatization, Fig. 5.

Even if the two cultivars behaved differently in micropropagation stages, statistical analysis showed that the results obtained with Dar 8 and Dar 24 cultivars were better than those obtained with Darrow cultivars.

4. **Conclusions**

The results in this experiment allow concluding:

- blackberry cultivars Dar 8 and Dar 24 can be propagated *in vitro* quite effectively.
- for culture initiation and multiplication of cultivars Darrow, Dar 8 and Dar 24 we recommend the culture medium containing major and minor salts as in Murashige and Skoog (1962) (MS) and Linsmaier - Skoog (1965) (LS) vitamins, supplemented with 0,3 mg/l N6 - benzilaminopurine (BAP), 0.1 mg/l gibberellic acid (GA_3) and 0,001 naphtylacetic acid (NAA).
- medium with MS reduced at 1/2 mineral salts, and LS vitamins, with 0. 1 mg/l IBA and 0.1 mg/l GA_3 was very good medium for rooting all three blackberry cvs..
- in all stages of micropropagation cultivars Dar 8 and Dar 24 had a better behavior than Darrow cultivar.

Acknowledgements

This paper was supported by MADR, Sectorial Plan ADER 2020, Project ADER 2.2.7. Contract 227/2011.

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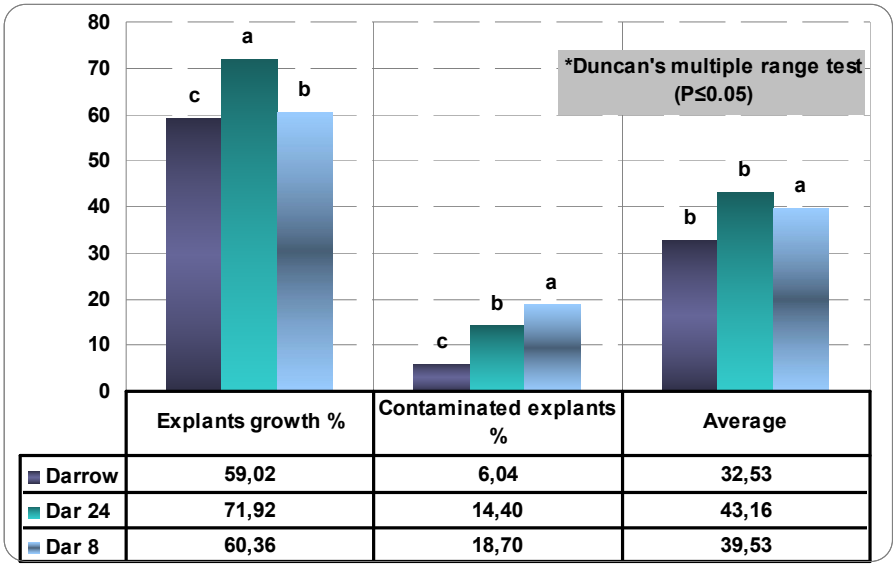
Tables

Table 1. Biological and DAS – ELISA serological testing results

Soiul	Biological methods								Phytosanitary status
	BRNV	RLMV	RLSV	RYNV	RYSV				
Darrow	-	-	-	-	-				Virus free
Dar 8	-	-	-	-	-				Virus free
Dar 24	-	-	-	-	-				Virus free
	DAS – ELISA								
	ArMV	RpRSV	SLRSV	TBRV	CMV	ApMV	CLRV	RBDV	
Darrow	-	-	-	-	-	-	-	-	Virus free
Dar 8	-	-	-	-	-	-	-	-	Virus free
Dar 24	-	-	-	-	-	-	-	-	Virus free

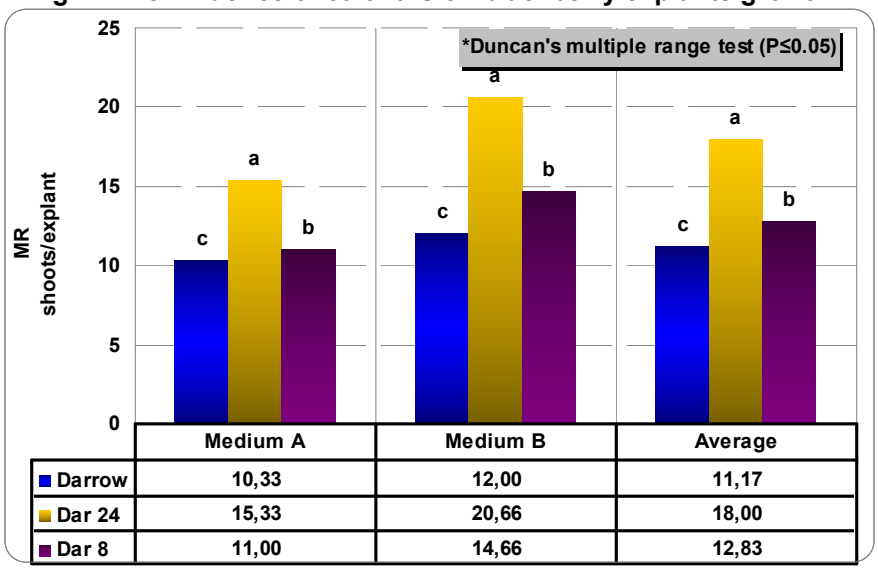
Nota: - = liber de virus

Figures



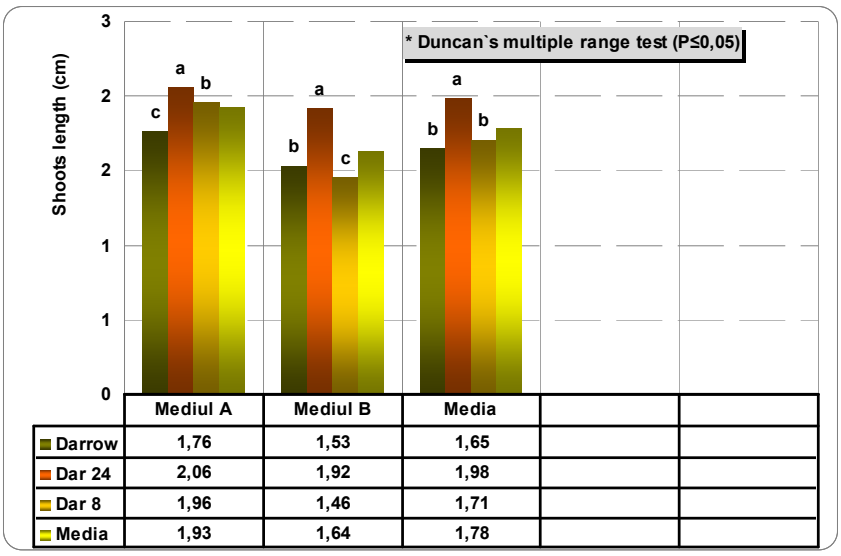
	Explants growth %	Contaminated explants %	Average
Darrow	59,02	6,04	32,53
Dar 24	71,92	14,40	43,16
Dar 8	60,36	18,70	39,53

Fig. 1. The influence of cultivars on blackberry explants growth



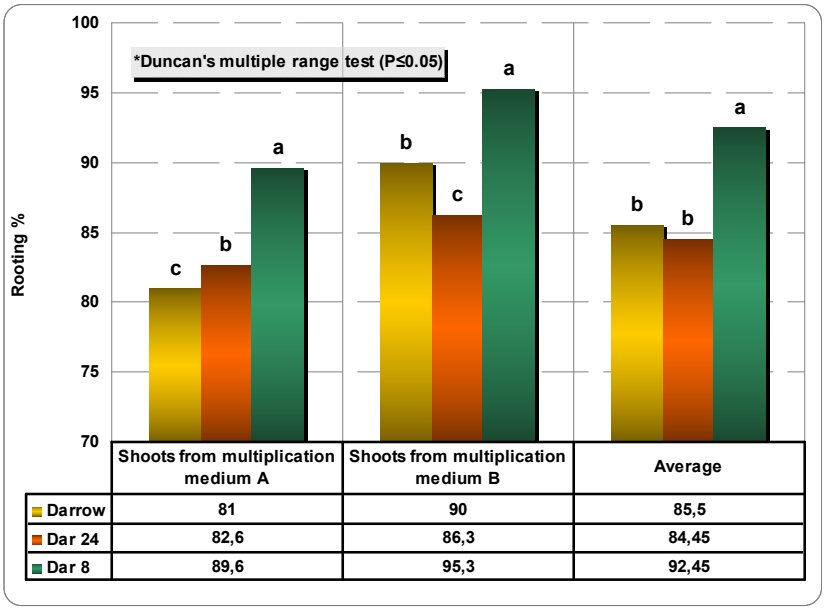
	Medium A	Medium B	Average
Darrow	10,33	12,00	11,17
Dar 24	15,33	20,66	18,00
Dar 8	11,00	14,66	12,83

Fig. 2. The multiplication rate (MR) of blackberry thorns cultivars after three subcultures



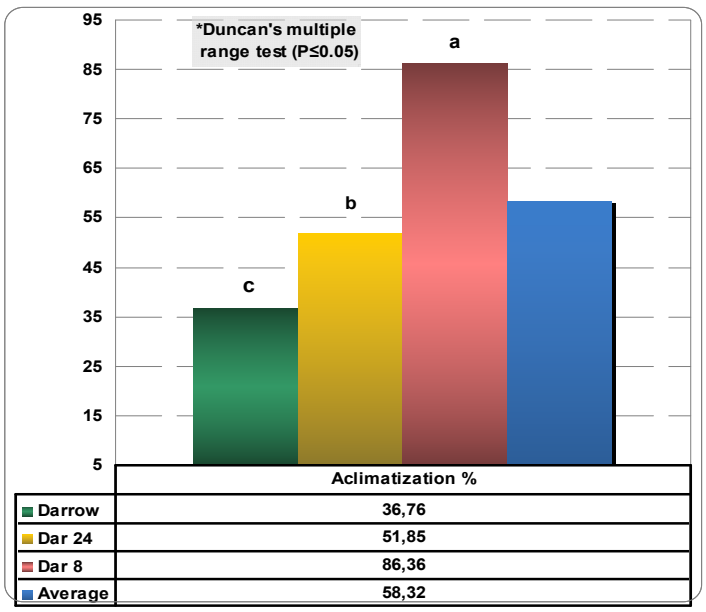
	Mediul A	Mediul B	Media		
Darrow	1,76	1,53	1,65		
Dar 24	2,06	1,92	1,98		
Dar 8	1,96	1,46	1,71		
Media	1,93	1,64	1,78		

Fig. 3. Shoots length of thorn blackberry cultivars after three subcultures on multiplication media



	Shoots from multiplication medium A	Shoots from multiplication medium B	Average
Darrow	81	90	85,5
Dar 24	82,6	86,3	84,45
Dar 8	89,6	95,3	92,45

Fig. 4. Behavior of thorn blackberry cultivars in the *in vitro* rooting depending on the shoots multiplication medium



	Aclimatization %
Darrow	36,76
Dar 24	51,85
Dar 8	86,36
Average	58,32

Fig. 5. Blackberry thorns cultivars plantlets aclimatization

Fig. 6. Aspects regarding blackberry micropropagation: A, B - multiplication and rooting stages; C, D – acclimatization; E, F – fortified plants