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Micropropagation of service tree (*Sorbus domestica* L.): role of some factors on *in vitro* proliferation and rooting, and *extra vitro* acclimatization

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Abbreviations: BA: 6-benzylaminopurine; IBA: indole-3-butyric acid

INTRODUCTION. – For centuries service tree (*Sorbus domestica* L.) has been propagated by seed (BIGNAMI, 1998) and as a result it is expressing a broad genetic variability in different aspects of plant, fruit and wood. As a fruit plant it has been subjected to a minimum selection and only occasionally the most interesting features were fixed through agamic propagation. Regarding service tree as timber species, there are no cultivars selected for this purpose although its timber is more valuable than the walnut (WOLF, 1999). Service tree belongs to fruit plants that grow naturally and spontaneously in the Mediterranean area; the valuable genetic heritage of the service tree is likely not only to remain unused but it could be even at risk of extinction due to its lack of competitiveness.

At the Faculty of Agriculture of University of Milan is in progress, for nearly a decade, *S. domestica* L. tree selection activity for timber production. Some plants have also been selected for high valuable fruit quality (PIAGNANI *et al.*, 2011), and among them two lines are candidates for triple aptitude plants: in fact, due to their erect and majestic bearing, make them suitable to urban decor. Selected plants can be used to replenish the lowland forests that act as an inverse buffer zone (river de-pollution) (HEFTING and DE KLEIN, 1998) and contribute to ecological connectivity, allowing the movement and retreat of animal and plant species (MCHUGH and THOMPSON, 2011), and representing opportunities for recreation and leisure.

The use of *in vitro* culture, as a tool of vegetative propagation, contributes to both the large scale production of interesting genotypes and

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the exploitation of any carry over effects of the technique particularly in term of increased vigour of the *extra vitro* plants. Increased vigour, sometimes showed by micropropagated plants, is an appreciated characteristic for timber production tree (FRANCLET, 1983). Moreover, it is providing a tool for long term preservation of genetic resources. During previous trials (PIAGNANI and BASSI, 2000) it was noted that, among the different stages of service tree micropropagation, the most critical were rooting and acclimatization. The importance of the growing media used in the acclimatization is often overlooked in scientific reports. However, some physical and chemical properties of propagation substrates, such as air space, available water content, pH and electrical conductivity, may directly influence rooting response (LANDIS, 1990). The aim of this research was to determine, for each selected plant line, the conditions to get the best performances both *in vitro* and *extra vitro* conditions. In particular it was investigated the role of different levels of BA and IBA in proliferation and rooting and the carryover effect of these two growth regulators and of different growing media on *extra vitro* growth.

MATERIALS AND METHODS. – In vitro *experiment*. *Plant material.* – A seedling orchard of *Sorbus domestica* L. was established in 2001 at the Experimental Farm (Cascina Baciocca, located in Cornaredo, Milan, Italy) of the University of Milan. Trees were spaced at 6.0 m × 6.0 m in a medium fertility sandy soil. Five seedlings lines of *S. domestica* L. generated from a single isolated plant, located in Bologna Apennines (Central Italy) and named 'Tosca' (lines 'T101', 'T103', 'T105', 'T1013' and 'T1016'), have been considered for the experiments of this research work. Lines were selected for timber production (vigorous, fast growing, upright habit). The selected lines 'T103' and 'T105' resulted to be scab tolerant and with erect and majestic bearing, therefore they could be suitable candidate to urban decor (parks and gardens). Moreover, the line 'T103' shows high fruit quality character.

Cultures, unless otherwise specified, were routinely maintained in growth room at 22-24°C, under cool white fluorescent lamps (Philips TDL 36W/33) with an irradiance of about 30 μmol s^{-1} m^{-2} (white light) and at 16-h photoperiod.

We supposed that BA carry over effect as well as IBA level used for rooting treatment could have a specific interaction with each genotype, consequently, they have been tested for both rooting and *extra vitro* experiments.

Explant establishment experiment. – In this trial we wanted to test the influence of the starting material (rejuvenated) on explant sterilization and growth. Two-year-old micropropagated plants from the selected lines 'T101', 'T105', 'T1013' and 'T1016'were considered. In the first decade of April and second decade of June (only for 'T1013'and 'T1016'), apical and lateral shoots were sampled from actively grown donor plants cultivated in pots. Shoots were leaf deprived and small single bud-cuttings were sterilised with $HgCl_2$ (0.5‰) for 15 min, rinsed three times with sterile distilled water and then cultured in 30 cc plastic vessels (Coulter cups, Thermo-Labs, Italy) with 10 ml of hormone free MURASHIGE and SKOOG (1962) minerals based medium, vitamins

as described by NITCH and NITCH (1969), Plant Agar (Duchefa, The Netherlands); pH was adjusted at 5.5 before autoclaving. Not contaminated explants were transferred to the same medium with 2.5 μM BA added. Number of not contaminated and proliferating explants was recorded after 21 days of culture.

Proliferation experiment. – 'T101', 'T105', 'T1013' healthy shoots, transferred to the same medium added with BA 2.5 μM, were sub-cultured any 21 days. At the end of the third subculture, shoots were used to assess the role of BA, at concentrations 1.25, 2.5 and 5.0 μM, on the proliferation coefficient. Explants were cultured into Linfa-boxes (Micropoli, Italy) with 100 ml medium. A total of 30 explants (2 Linfa-boxes) per each plant line and BA concentrations were used in the first subculture, maximum 4 Linfa-boxes were used in sub-sequent subcultures. Each subculture lasted 4 weeks and after each subculture shoot number was recorded. Three subcultures were considered.

Rooting experiment. – We suppose that the carry over effect of BA could have an interaction with then genetic background of each line. Therefore, to test this hypothesis three IBA concentrations, 1000, 2000 and 3000 mg l^{-1} were used on shoots propagated from two BA concentrations (2.5 and 5.0 μM). For this rooting experiment and for each plant line 40 shoots per each BA × IBA level were used. Four Linfa-boxes containing ten shoots were used, each Linfa-box was considered one replication.

Extra vitro *acclimatization experiment. Plant material*. – Shoots of 1.0-1.5 cm in length with visible root primordia were transplanted onto 5 cm plastic jars with holes filled with different growing media and transferred to mini greenhouses (52 × 34 cm) with transparent lids. Plants were kept under observation until both apex growth and roots re-growing out the drainage hole were noticed; the number of days elapsed from the time of transplantation was recorded. About three months later, the percentage of survival plants was calculated.

Growing media. – Growing media (GM) were chosen on the basis of the parameters that mainly affect seedling growth: pH, aeration, water holding porosity, available nutrients content and potential disease suppressiveness capability. Two types of GM with different water/air porosity were chosen: two fine textured materials (S1 and S2) differently limed and fertilized, and a coarse textural material one (S3). S1 is a fine mixture of Baltic white peat (55% v/v), 3-6 mm perlite (25% v/v) and Baltic brown peat (20% v/v); S2 is a fine mixture of Baltic white peat (30% v/v), Baltic brown peat (30% v/v), German black peat (20% v/v), Irish brown peat (20% v/v); S3 is a mixture of coarse white peat (30% p/p), perlite (30% v/v), dark brown peat (25% v/v) and green compost (15% v/v). GM were also evaluated (3 replicates/sample) for the following physical, hydrological and chemical properties, according to the European Standards for Soil Improvers and Growing Media: dry bulk density, total porosity, air volume and water volume percentage at different pressure suction (pF 1.0 and pF 1.7) and easily available water (UNI EN 13041:2012); pH (UNI EN 13037:2012); electrical conductivity (UNI EN 13038:2012); water soluble nutrients (UNI EN 13652:2001); Mg, Ca and K were detected on 1:5 v/v water extracts by ICP MS spectrometry and NH_4-N, NO_3-N and PO_4-P by colorimetric methods (ISO, 2005; ISO, 1996; MURPHY and RILEY, 1962).

Growing media effect on plant growth was tested by 3 bioassays: a) germination test in Petri dish (DE BERTOLDI and ZUCCONI, 1983) in which germination and early roots elongation of cress seeds (*Lepidium sativum* L.) kept for 3 days in water extracts of GM, were compared with those kept in pure water (4 replicates/sample, 10 seeds/Petri dish); b) root elongation test in pot (ISO 11269-1:2012) in which roots length of pre-

germinated seeds of barley (*Hordeum vulgare* L.), grown for 5 days in tested GM, were compared with those grown on a control consisting of sand (3 replicates/sample, 6 seeds/pot); c) growth test in pot (ISO 11269-2:2012) in which fresh and dry aerial biomass of lettuce (*Lactuca sativa* L.), cultivated for 30 days on the tested GM, were compared with those grown on a lightly fertilized standard medium (4 replicates/sample, 3 plants/pot). Before beginning the *extra vitro* acclimatization experiment, all GM were sterilized by autoclaving (121°C, 15 min); the effects of the heat treatment on physical, hydrological and chemical properties and on responses to bioassays were checked.

Statistical analysis. – *In vitro* experiments were analysed for statistical significance via factorial univariate analysis of variance (SPSS ver. 19). When calculated values for F were significant, the Tukey's t test was used to interpret significant differences among the means. All per cent values were arcsin transformed and were subjected to Kolmogorov-Smirnov non parametric test of normality: percentage data which did not passed the test were analysed by using the $\chi 2$. Acclimatization data were treated as rank transformation procedures (Conover and Iman, 1981) a part for those of survived plants that were analysed by using the $\chi 2$. Otherwise stated statistical analysis was performed at 0.05 level. Data of growing media properties before and after the heat treatment were subjected to Student's t-test; growing media characterization was analysed via one way ANOVA (SPSS ver. 19). The significant differences among the mean values were calculated following Tukey's t test at 0.05 level.

Results. – *Explant establishment*. – Making explants in April 100% sterile microcuttings were obtained and moreover all those explants started proliferating. Postponing explants two months later caused sterile percentages dropping down to 50% and 60% for line 'T1013' and line 'T1016', respectively (data not shown). Regarding June explants proliferation the two tested genotypes behaved differently, in fact, 'T1013' explants started becoming brownish and finally died, while 58.3% of 'T1016' explants were able to proliferate.

Proliferation. – As from Table 1, two lines out of three were stimulated from the highest (5.0 μM) BA concentration; on the contrary, line 'T1013' was not affected by BA level. The highest proliferation coefficient was observed in explants of 'T105' line cultured at BA 5.0 μM, whereas the lowest values was detected in explants of 'T1013' line cultured on 1.25 μM BA. Independently from BA level, 'T105'and 'T101'lines have got the highest proliferation coefficient, 2.3 and 2.2, respectively. In respect to the effect of subculture, independently from all the other factors, the third subculture scored the highest proliferation coefficient, 2.4 (data not shown).

Rooting. – In Figure 1 only the statistically significant interactions are reported. Independently from IBA treatment, only line 'T1013' was affected by BA level used in the previous phase (Fig. 1A). Shoots belonging to plant line 'T101', from 2.5 μM BA, scored the maximum rooting,

TABLE 1. – *Interaction among plant line and BA level on the proliferation coefficient.*

Plant line	BA	Proliferation coef. (n)	
T'101'	1.25	1.4	a
	2.50	2.1	b
	5.00	2.8	c
T'105'	1.25	0.7	a
	2.50	2.2	b
	5.00	3.5	d
T'1013'	1.25	1.1	a
	2.50	1.4	a
	5.00	1.6	a

Means (from two experiments) with the same letters are not significantly different according to the Tukey's test, $30 \leq n \leq 60$ ($p = 0.05$). BA: 6-benzylaminopurine.

76.7%, the minimum, 37.5%, was scored by 'T1013' shoots coming from 5.0 μM BA. Contrariwise, all plant lines were affected by IBA level at a different extent: the lowest concentration, 1000 mg l^{-1}, improved plant selection 'T101'and 'T1013' rooting, on the contrary, 'T105' was significantly stimulated by the highest level, 3000 mg l^{-1} (Fig. 1B).

Acclimatization. – Growing media properties. – All the tested materials (Table 2A) showed high total porosity, that is unequally partitioned among macro and micro pores. At pF 1.0 in S1 and S2 the water-holding porosity (micro pores) represented the majority of total porosity while in S3 a large part of porosity was occupied by air and a low easily available water was observed. The fine textured GM S1 and S2 had a quite

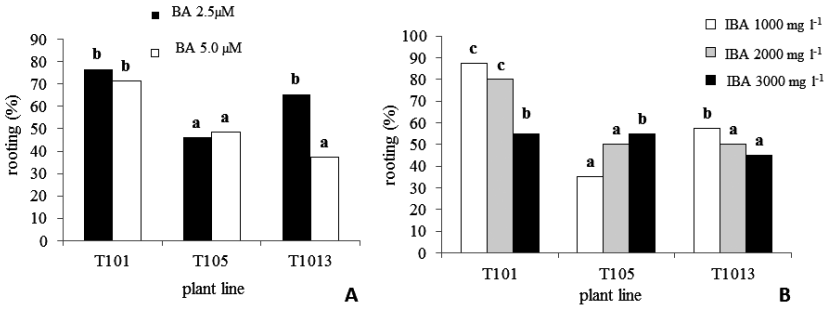

FIG. 1. – Effect of interaction between plant lines x BA (A) and plant lines x IBA (B) on rooting percentage. Units: BA = μM, IBA = mg l^{-1}. Means with the same letters are not different according to the Tukey's test ($n = 10$, $p = 0.05$).

TABLE 2. – *Physical, hydrological and chemical characteristics (A) of growing media, and results of bioassays (B) expressed as the ratio between growing media and control data.*

			S1		S2		S3	
A)								
	Dry bulk density	(kg m^3)	115	a	131	a	249	b
	Total porosity	(%, v/v)	93.3		92.3		87.9	ns
	Water vol. pF 1.0	(%, v/v)	83.1	b	85.3	b	55.1	a
	Air vol. pF 1.0	(%, v/v)	10.2	a	7.00	a	32.8	b
	Water vol. pF 1.7	(%, v/v)	47.1		46.4		41.2	ns
	Air vol. pF 1.7	(%, v/v)	46.2	a	46.8	a	52.1	b
	EAW	(%, v/v)	36.0	b	38.9	b	13.9	a
	pH		5.75	a	6.24	b	6.74	c
	EC	(dS/m)	0.33	a	0.51	b	0.97	c
	Water sol. NO_3-N	(mg/L GM)	93.5	a	127	b	305	c
	Water sol. NH_4-N	(mg/L GM)	1.20		1.00		2.25	ns
	Water sol. Mg	(mg/L GM)	80.5	b	127	c	59.5	a
	Water sol. Ca	(mg/L GM)	2990	a	5400	c	3540	b
	Water sol. K	(mg/L GM)	86	a	101	b	490	c
	Water sol. P	(mg/L GM)	27.7	a	25.8	a	48.0	b
B)								
	Germination test		0.68		0.72		0.61	
	Root elongation test		1.33		1.34		0.79	
	Growth test		28		30		17	

Values with the same letters in each line are not significantly different according to the Tukey's test (p = 0.05). EAW: easily available water (water volume content between pF 1.0 and pF 1.7); GM: growing media.

similar chemical profile (Table 2A). Nevertheless, due to lime addition, S2 had a higher pH and higher contents of calcium and magnesium that, together with nitrate and potassium contents, enhanced the salinity of the aqueous extract. In S3 the presence of compost entails a great content of water soluble nutrients and a consequent high value of the electrical conductivity. The use of plant bioassays to describe the quality of GM represent a new and concise approach to evaluate in short time the capability of substrates to support plant cultivation in soilless culture (ZACCHEO *et al.*, 2009). The Germination Indexes obtained by the studied GM (Table 2B) were found to be over the critical value of 0.6 (risk of toxicity with temporary inhibition of growth). The measurements of barley root length in S1 and S2 were over the control while S3 induced a reduction in the root length. In growth test with lettuce all the samples supported greater productions than the control.

Sterilization is a treatment not always applied to growing media used in *extra vitro* acclimatization; in our lab we have adopted the procedure to sterilize GM because in previous trials it was observed that, although agar was washed off the root system, in non-sterile media young plantlets were smothered by the mycelia mat. The physical and hydrological properties of GM were not significantly influenced by the sterilization process and the results of elongation and growth bioassays repeated on sterilized samples did not differ from those obtained before autoclaving. On the other hand, heat treatment had an influence on growing media pH, EC, soluble ammonium and on the response to germination test (Fig. 2): the Germination Indexes of S1 and S2 slightly decreased while in S3 it increased from 0.60 to 0.80, probably due to lowered salinity. In Figure 3 the standardized data of the three sterilized GM are reported

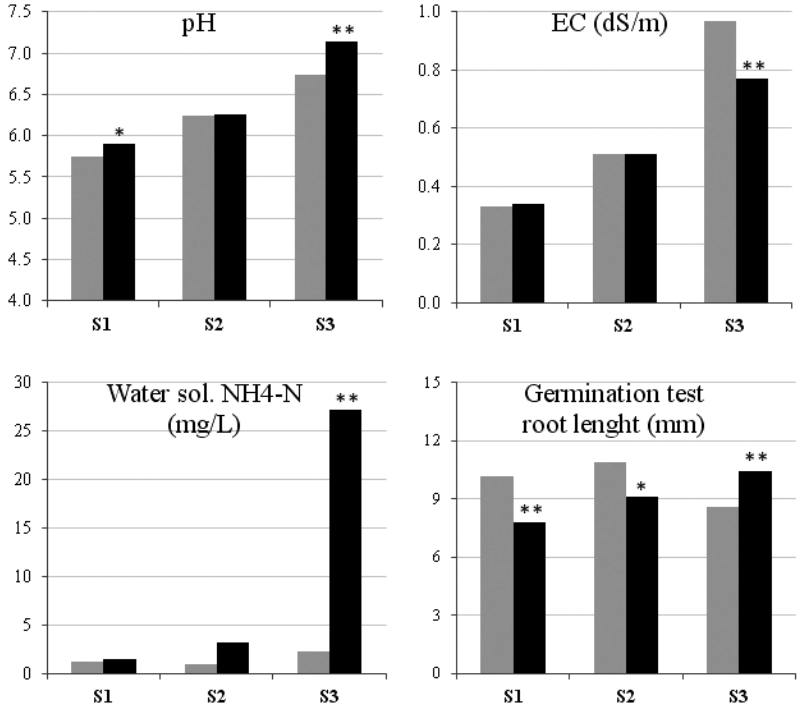

Fig. 2. – Effect of sterilization on growing media pH, electrical conductivity (EC), water soluble ammonium and germination test (untreated, grey bars; treated, black bars). Student t test: *, ** for p = 0.05 and p = 0.01, respectively.

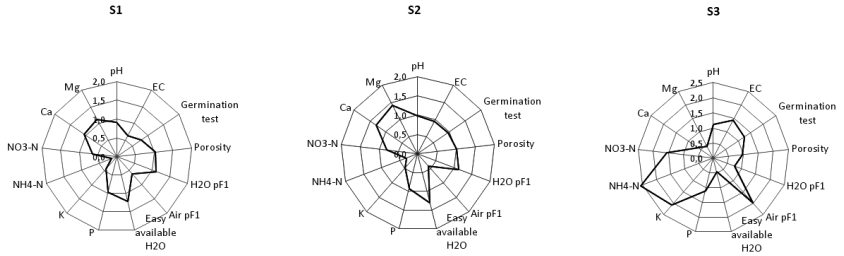

FIG. 3. – Radar charts from the standardized values of the principal properties of growing media before sterilization.

in radar graphs. The fine textured S1 and S2 were quite similar, only differing in the amount of basic cations and consequently in pH and electric conductivity; the presence of compost and perlite makes S3 very different from the other GM in its physical and chemical properties: high aeration, low available water and richness in nutrients.

Extra vitro *plant adaptation*. – *Extra vitro* plant adaptation was measured through the days occurred to the plant to activate the apex and root growth in *extra vitro* condition. In Table 3 are shown data recorded for plant line × BA level x medium in terms of average days for apex growing. Only plant line 'T105' was affected by BA level used in the proliferation phase. The best results, independently on BA level, were obtained on S3 medium, on the opposite the worst results, 25.4 and 27.8 days, were scored on S1 medium if shoots came from 2.5 μM BA otherwise on S2 medium if the shoots come from 5 μM BA. Finally, regardless both BA level and medium, line 'T1013' duplicates the timing of apex growth compared to plant line 'T101'.

Table 4 (A, B) shows interaction values for the tested factors, plant line x IBA level (A) and plant line × BA level (B) in terms of days occurred to roots to appear out of the drainage holes. In this case there wasn't any interaction plant line x medium. Plant line 'T101' gave the best performances (12.3 and 13.9 days) together with line 'T105' (17.8 days) coming from 2.5 μM BA. The worst result, 27.7, was scored with plant line 'T105' coming from 5 μM BA. In respect to plant line × IBA level interaction plant line 'T101' was not affected by IBA level on the contrary plant lines 'T1013' and 'T105' gave the best performances by using 1000 and 2000 mg l^{-1} respectively. Three months later the number of survived plants was recorded and surprisingly, and independently on the other considered factors, plants grown on S3 medium scored the

TABLE 3. – *Interaction among plant line, BA level and growing media on days for apex* extra vitro *growth.*

Plant line	BA (μM)	GM	Average days	
101	2.5	S1	9.3	a
		S2	9.2	a
		S3	9.6	a
	5.0	S1	8.5	a
		S2	8.5	a
		S3	11.8	a
1013	2.5	S1	19.9	b
		S2	21.0	b
		S3	19.1	b
	5.0	S1	17.7	b
		S2	17.6	b
		S3	20.4	b
105	2.5	S1	25.4	c
		S2	20.1	b
		S3	15.3	a
	5.0	S1	18.3	b
		S2	27.8	c
		S3	10.1	a

Means with the same letter are not significantly different according to the Tukey's test, $4 \leq n \leq 14$ ($p = 0.05$). BA: 6-benzylaminopurine; GM: growing media.

minimum frequency of survived plants (37.3%) while at the opposite there is S1 medium which scored the maximum frequency (71.2%) (Table 5). Plant line 'T101, independently on BA and IBA level, scored the maximum survival on S1 (85.0%). Plant lines 'T105' and 'T1013', scored the maximum plant survival rate on S1, respectively 64.9% and 67.1%, only if such plants came from 2.5 μM BA. In general for these two last lines the role of IBA on plant survival was significant only if the plants came from 2.5 μM BA.

DISCUSSION. – *In vitro* culture induces rejuvenation (MULLINS *et al.,* 1979) and considering the establishment results from this research our lines keep this feature at least up to two years from their transferring to *extra vitro* conditions. Results from our previous experiments (unpublished data) were considering explants belonging to 10 years old plant lines; only 30-40% of such explants began proliferating in 6-8 weeks but

TABLE 4. – *Interaction between plant line and IBA level (A), and plant line and BA level (B) on days of growth occurring to roots to appear out of the drainage holes.*

		Plant line								
		T101			T1013			T 105		
A)	IBA (mg l^{-1})	1000	2000	3000	1000	2000	3000	1000	2000	3000
	Average days	14.2 a	16.4 a	8.7 a	26.9 b	16. 9 a	24.4 b	16.5 a	22.2 b	18.0 ab
B)	BA (μM)	2.5	5.0	-	2.5	5.0	-	2.5	5.0	-
	Average days	12.3 a	13.9 a	-	20.3 b	17.5 b	-	17.8 a	27.7 c	-

Means with the same letter are not significantly different according to the Tukey's test, $4 \leq n \leq 14$ ($p = 0.05$). BA: 6-benzylaminopurine; IBA: indole-3-butyric acid.

moreover up to 70% of plant material was contaminated. In this research the explants belonged to two-year-old micropropagated plants and we got not only 100% of sterile microcuttings but shoots started proliferating within 3 weeks. It is known from the literature that difficult to propagate *Pyrus* rootstocks rooted better when cuttings belonged to micropropagated plants than from conventionally produced plants (JONES and WEBSTER, 1989). Nevertheless it was observed that the physiological state of the donor plant at the time of explants excision had a predominant role affecting both sterilization efficiency and proliferation (MURASHIGE, 1974).

Proliferation coefficients resulted to be different among lines to confirm our hypothesis that service tree has been propagated by seed for many centuries and, given the likely close allogamy, quite wide variability (heterozygous) it was expected for different plant traits, which affected the *in vitro* performance of each line. One of the most important factors affecting *in vitro* adventitious rooting is the choice of auxin, its concentration and duration of the tissues exposition (DE KLERK *et al.,* 1999). One problem related to *in vitro* rooting is the difficulty of inducing a root system which can support plant growth once it is transferred to *extra vitro* conditions. Adventitious roots produced *in vitro* may present abnormalities that depends on the treatments under which they were developed: high auxin levels, for instance, confer an abnormal morphology and function (PIAGNANI and BASSI, 2000). Our results confirm these statements because the highest IBA tested level not only reduced rooting efficiency but caused, in most cases, plant loss during acclimatization. BA which is the most widely used cytokinin for

TABLE 5. – *Interaction among plant lines, BA level, IBA level and growth media on percentage of survived plants.*

Plant line	BA (μM)	IBA (mg l^{-1})	S1 (%)		S2 (%)		S3 (%)	
101	2.5	1000	91.3		87.0		72.0	ns
		2000	68.2		60.9		40.9	ns
		3000	40.0		20.0		11.8	ns
			70.0	**c**	**60.7**	**b**	**45.3**	**a**
	5.0	1000	72.7		68.2		45.8	ns
		2000	95.0		84.2		66.7	ns
		3000	88.9		66.7		46.7	ns
			85.0	**c**	**73.2**	**b**	**53.3**	**a**
	2.5+5.0		**77.5**	**c**	**66.6**	**b**	**50.8**	**a**
105	2.5	1000	90.9	b	81.8	b	46.7	a
		2000	92.3	b	69.2	b	46.7	a
		3000	41.7	b	53.8	b	7.7	a
			75.0	**c**	**67.6**	**b**	**30.8**	**a**
	5.0	1000	50.0	b	71.4	b	11.1	a
		2000	58.3		75.0		23.1	ns
		3000	55.6		58.8		22.2	ns
			55.3	**b**	**66.7**	**b**	**20.0**	**a**
	2.5+5.0		**64.9**	**b**	**67.1**	**b**	**25.3**	**a**
1013	2.5	1000	62.5	b	56.2	b	29.4	a
		2000	100	b	58.8	b	11.1	a
		3000	100	b	57.1	b	50.0	a
			87.2	**c**	**57.4**	**b**	**28.6**	**a**
	5.0	1000	54.5		72.7		27.3	ns
		2000	0.0	a	62.5	b	60.0	b
		3000	28.6		20.0		0.0	ns
			30.8		**58.3**		**33.3**	**ns**
	2.5+5.0		**67.1**	**b**	**57.7**	**a**	**30.3**	**a**
Gran total			**71.2**	**c**	**64.4**	**b**	**37.3**	**a**

Data were analysed by χ^2, 3 × 2 contingency tables (p = 0.05). Means with the same letter are not significantly different according to χ^2 test.

shoot multiplication in tissue culture (WERBROUCK *et al.*, 1996), on the contrary, has a controversial role: it may support rooting (PIAGNANI *et al.*, 1996) or can have an inhibitory action on both rooting (ADELBERG and NAYLOR-ADELBERG, 2012) and acclimatization (WERBROUCK *et al.*, 1996). Experimental trials (WERBROUCK *et al.,* 1996) show that reduction in rooting and acclimatization performances, observed in some plants cultured on BA-supplemented medium may result from production of an inhibitory BA metabolite 9G-BA. This metabolite accumulates at the base of plantlets *in vitro* and remains for more than 6 weeks. During acclimatization, again in accordance with our expectations, the response to BA and IBA was not unique for the three lines.

The physical characteristics of GM play a major role on their performances; in particular an efficient GM must have high pore space, essential for the supply of air and water to the root system. S1 and S2 showed high water availability and low aeration, suitable for plants with high water needs, but that could involve potential risk of oxygen depletion around the root system in case of too frequent high-dose irrigations (MICHEL, 2010); on the other hand, the well aerated S3 required frequent irrigation to provide the adequate quantity of water for plant needs.

Considering the aim of the bioassays adopted to evaluate the GM, the germination test with cress is specifically able to point out the presence of factors like organic and inorganic contaminants, herbicide residues, high salinity, organic metabolites, that may cause toxicity in the early stage of plant development; root elongation test is sensitive to toxicants and to the root environment and the 30 days growth test with lettuce is useful to test nutrient supply to plants together with the potential phytotoxicity of GM. Comparing the results obtained by each GM in this set of bioassays, S1 and S2 showed a lack of toxicity and a good inherent fertility; in S3 no instantaneous toxicity came out, but some physical and/or chemical factors caused an inhibition of roots development and lower aerial biomass production than that achievable on the basis of available nutrients.

After autoclaving GM, a small increase of pH in S1 and a marked basification in S3 was observed, the latter due to a conspicuous release of ammonium related to the presence in S3 of labile organic compounds from composted yard wastes: presumably basification, inducing salts precipitation, was responsible of the decrease of electrical conductivity observed in S3, that positively influenced the results obtained in germination test applied to autoclaved GM.

The *extra vitro* acclimatization results show that growing media have great impact of on the performance of young plantlets of service. Our data suggest that the development of a root system is promoted by growing media which guarantee both high water retention capacity and easily available water, allowing the maintenance of a liquid interface between roots and solid surfaces. The different performance of S1 and S2 GM, having analogous water retention characteristics, suggests that service trees plantlets are sensitive to narrow differences in growing media salinity. The use of a coarse material with high air capacity like S3 had an overall negative effect on acclimatization, with a low percentage of plant survival (30%). Moreover, the presence of compost in the mixture as a peat partial substitute could be a further element of risk due to the low stability of organic matter and the abundance of soluble salts. Nevertheless, compost can add to substrates for nurseries a suppressive capacity against harmful microorganisms (Raviv, 2009), even though the effect of sterilization techniques on this property is yet to be investigated. The release of ammonium, the changes in pH and electrical conductivity and in the response to the germination tests observed in our experiments suggest the importance of performing chemical analysis and bioassays after the thermal treatment.

Conclusions. – Service tree has characterised by high heterozygosity and it could be evocated for explaining the high phenotypic variability observed both *in vitro* and in *extra vitro* trials. During this research we have found the conditions that make the studied lines to perform both *in vitro* and *extra vitro* environment. In addition we suggest the use of plant bioassays to evaluate the appropriateness of substrates for *ex vitro* acclimatization in preliminary screening.

REFERENCES

Adelberg, J. and Naylor-Adelberg, J.: Effects of cytokinin on multiplication and rooting of *Aloe barbadensis* during micropropagation on agar and liquid medium. *Journal of Medicinally Active Plants 1,* 1-5 (2012). Available at scholarworks.umass.edu/jmap.

Bignami, C.: Il sorbo. In: *Conservazione dei fruttiferi minori*. Proceedings of the National Workshop on EC Project GENRES 29 (Dept. of Horticulture, University of Florence, 27-28 November 1998), p. 18 (1998). Available at http://www.unifi.it/ueresgen29.

Conover, W.J. and Iman, R.L.: Rank transformations as a bridge between parametric and nonparametric statistics. *Am. Stat. 35,* 124-129 (1981).

De Bertoldi, M. and Zucconi, F.: Analisi della fitotossicità della sostanza organica in decomposizione mediante biosaggio di *Lepidium sativum* (Phytoxicity test of decomposing organic substance using *Lepidium sativum*). *Quad. Ist. Ric. Acque 64*, 1-3 (1983).

De Klerk, G.J., Van der Krieken, W. and De Jong, J.: Review the formation of adventitious roots: New concepts, new possibilities. *In Vitro Cell. Dev.-Pl. 35,* 189-199 (1999).

DURKOVIC, J. and MISALOVA, A.: Wood formation during *ex vitro* acclimatisation in micro propagated true service tree (*Sorbus domestica* L). *Plant Cell Tiss. Org. 96,* 343-348 (2009).

FRANCLET, A.: Rejuvenation: theory and practical experiences in clonal silviculture. In: *The 19th Meeting of the Canadian Tree Improvement Association* (L. Zsuffa, R.M. Rauter, C.W. Yeatman, eds). Proceedings of the Biannual Meeting (Toronto, Canada, August 22-26 1983). Canadian Forestry Service, Environment Canada, for the Canadian Tree Improvement Association, Ottawa, Canada, pp. 96-134 (1984). ISBN 0662138813.

HEFTING, M.M. and DE KLEIN, J.J.M.: Nitrogen removal in buffer strips along a lowland stream in the Netherlands: a pilot study. *J. Environ. Pollut. 102,* 521-526 (1998).

ISO 13395:1996: Water quality - Determination of nitrite nitrogen, nitrate nitrogen and the sum of both by flow analysis (CFA and FIA) and spectrometric detection (1996).

ISO 11732:2005: Water quality - Determination of ammonium nitrogen - Method by flow analysis (CFA and FIA) and spectrometric detection (2005).

ISO 11269-1:2012: Soil quality - Determination of the effects of pollutants on soil flora - Part 1: Method for the measurement of inhibition of root growth (2012)

ISO 11269-2:2012: Soil quality - Determination of the effects of pollutants on soil flora - Part 2: Effects of chemicals on the emergence and growth of higher plants (2012).

JONES, O.P. and WEBSTER, C.A.: Improved rooting from conventional cuttings taken from micropropagated plants of *Pyrus communis* rootstocks. *J. Hortic. Sci. 64*, 429-434 (1989).

LANDIS, T., TINUS, R.W., MC DONALD, S.E. and BARNETT, J.P.: The container tree nursery manual. In: *Containers and Growing Media*. Agric. Handbook 674. U.S. Department of Agriculture Forest Service, Washington D.C., pp. 41-85 (1990).

MCHUGH, N. and THOMPSON, S.: A rapid ecological network assessment tool and its use in locating habitat extension areas in a changing landscape. *J. Nat. Conserv. 19,* 236-244 (2011).

MICHEL, J.C.: The physical properties of peat: a key factor for modern growing media. *Mires and Peat 6,* art. 02, 1-6 (2010). Available at http://www.mires-and-peat.net (accessed Apr 09, 2010).

MULLINS, M.G., NAIR, Y. and SAMPET, P.: Rejuvenation *in vitro*: induction of juvenile characters in an adult clone of *Vitis vinifera* L.. *Ann. Bot. 44,* 623-627 (1979).

MURASHIGE, T.: Plant propagation through tissue cultures. *Annu. Rev. Plant Physiol. 25,* 135-166 (1974).

MURASHIGE, T. and SKOOG, F.: A revised medium for rapid growth and bioassays with tobacco cultures. *Physiol. Plant. 15,* 473-497 (1962).

NITSCH, J.P. and NITSCH, C.: Haploid plants from pollen grains. *Science 163,* 85-87 (1969).

PIAGNANI, C. and BASSI, D.: Aspetti della propagazione di *Sorbus* spp. (*In vivo* and i*n vitro* propagation of *Sorbus spp.* from juvenile material). *Italus Hortus 5*, 3-7 (2000). (abstract in English).

PIAGNANI, C., ZOCCHI, G. and MIGNANI, I.: Influence of Ca^{2+} and 6-benzyladenine on chestnut (*Castanea sativa* Mill.) *in vitro* shoot-tip necrosis. *Plant Sci. 118*, 89-95 (1996).

PIAGNANI, M.C., DEBELLINI, C. and LO SCALZO, R.: Phyllometry and carpometry, chemical and functional characterization of fruits of *Sorbus domestica* L. (service tree) selections. *J. Berry Res. 1,* 1-16 (2012). DOI:10.3233/BR-2011-023.

RAVIV, M.: Recent advance in soil-borne disease control using suppressive media. *Acta Hortic. 819*, 125-134 (2009).

UNI EN 13652:2001: Soil improvers and growing media – Extraction of water soluble nutrients and elements (2001).

UNI EN 13041:2012: Soil improvers and growing media - Determination of physical properties - Dry bulk density, air volume, water volume, shrinkage value and total pore space (2012).

UNI EN 13037:2012: Soil improvers and growing media - Determination of pH (2012).

UNI EN 13038:2012 – 2012c Soil improvers and growing media - Determination of electrical conductivity (2012).

WERBROUCK, S.P.O., STRNAD, M., VAN ONCKELEN, H.A. and DEBERGH, P.: Meta-topolin, an alternative to benzyladenine in tissue culture? *Physiol. Plant. 98,* 291-297 (1996).

WOLF, H., ARENHÖVEL, W., BEHM, A., FRANKE, A., KLEINSCHMIT, J., ROGGE, M., SCHNECK, D., SCHNECK, V., SCHULZKE, R. and TABEL, U.: Conservation and breeding of wild fruit tree species in forestry. *Acta Hortic. 538,* 57-61 (2000).

ZACCHEO, P., CRIPPA, L. and ORFEO, D.: Application of ISO 112-69-1 root elongation bioassay for testing the physical properties of growing media. *Acta Hortic. 819*, 427-434 (2009).

SUMMARY. – The use of *in vitro* culture, as a tool of vegetative propagation, contributes to both large scale multiplications of interesting genotypes. Regards explant establishment 100% of both sterile and proliferating explants were obtained cutting the explants from two years old micropropagated donor plants. Two selected lines out of three were stimulated in proliferation from the highest (5.0 μM) BA concentration. 1000 g l^{-1} IBA and 2.5 μM BA enhanced rooting in two selected lines out of three. Regarding *extra vitro* acclimatization the importance of growing media is often overlooked in scientific reports. Different types of growing media were tested, and the relationships among their chemical and physical properties and plant responses were investigated.