CARYOLOGIA Vol. 60, no. 1-2: 178-181, 2007

In vitro response of two Sicilian genotypes of *Morus* (L.) through axillary bud culture

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Abstract — A protocol for the *in vitro* establishment of two Sicilian genotypes of mulberry has been set up. Multiple shoots were initiated and plantlets were produced from buds collected from mature trees of *Morus alba* L. and *Morus nigra* L., grown in the field. The influence of two explanting times (September and December) of buds on the *in vitro* response has been observed.
The percentage of sprouting after four weeks of culture was greatly higher with material explanted from the field in September compared with the one collected in December. *Morus nigra* L. produced more vigorous shoots than *Morus alba* L.. After the second subculture the number of shoots produced decreased, in both genotypes. No differences were observed for both genotypes regarding the multiplication rate.

Key words: Axillary buds, *in vitro* culture, micropropagation, *Morus* (L.)

INTRODUCTION

Mulberry, typical woody plant of the Mediterranean basin, belongs to the *Morus* genus and to the *Moraceae* family. Among the species belonging to this genus, *Morus alba* L. (white mulberry) and *Morus nigra* L. (black mulberry) are of great relevance. Both species originated in Central and Oriental Asia, from where they spread through Europe at the beginning of sixteenth century. In the past, *Morus* L. cultivation was widespread in Central Italy and in Sicily, because of the mulberry foliage use for rearing silkworms (*Bombyx mori* L.). Nowadays, its cultivation has greatly decreased and it is mainly used as ornamental plant in gardens. Particularly, black mulberry is cultivated for the tasteful fruits, but also for its potential pharmaceutical and cosmetic use.

The recovery, characterization and the valorization of local ecotypes is a priority in the modern sustainable agriculture. Traditional and innovative methods are available for the preservation of the germplasm against the erosion of biodiversity. Among biotechnological methods, micropropagation can help to overcome some problems of large scale mulberry propagation, hindered by low rooting efficiency (Bhau and Wakhulu 2001).

Morus L. micropropagation has been achieved in several species, mainly through axillary bud proliferation (Bhau and Wakhulu 2003; Lu 2002; Pattnaik and Chand 1997; Tewary *et al.* 2000; Vijaya Chitra and Padmaja 1999; 2002; 2005; Wakhulu and Bhau 2000).

The present study was carried out to evaluate the response of two Sicilian local ecotypes of mulberry to *in vitro* culture and to establish an efficient protocol for their rapid micropropagation through axillary buds.

MATERIALS AND METHODS

One year old shoots were collected from about 80 years old trees of two Sicilian genotypes of *M. nigra* (L.), "Fontanarossa Nera" (FN) and of *M. alba* (L.) "Fontanarossa Bianca" (FB) grown in the field (Palermo province, Italy). For each genotype, a single tree provided the explants that were collected in two times: September and December. The explants containing axillary buds, cut into pieces 10-12 cm long, were surface sterilized by immersion for 10 minutes in liquid detergent, followed by immersions in 0.1% (w/v) fungicide solution, in 70% (v/v) ethyl alcohol for 3 min, in 20% (v/v) commercial bleach for 12 min and finally rinsed with autoclaved distilled water two times. From the surface-disinfected shoots, axillary buds were excised and scale leaves were re-

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moved. For each genotype, 50 buds were cultured in September and 80 buds in December. Five buds per Petri dish were placed vertically on the culture medium (Fig. 1). The medium used contained MURASHIGE and SKOOG mineral salts and vitamins (1962) added with 30 g/l sucrose, 500 mg/l ascorbic acid, 1.5 mg/l 6-benzylaminopurine (BAP) and 0.3 mg/l 2,4-dichlorophenoxyacetic acid (2.4 D). After the budbreaking, shoots obtained were subcultured every 45 days on the same medium. To induce rooting, after the second subculture, shoots were cultured on a medium containing MURASHIGE and SKOOG salts and vitamins (1962) added with 30 g/l sucrose, 0.5 mg/l indole-3-butyric acid (IBA) and 0.5 mg/l α-naphthalene acetic acid (NAA). The pH of the media was adjusted to 5.8 before gelling them with 8 g/l agar (Type washed, Sigma). Media were autoclaved at 104 kPa and 121°C for 20 min. All the cultures were incubated at 27±1°C, under cool white fluorescent lamps (Philips TLM 30W/84, France) with a photosynthetic photon flux density of 35 μmol m^{-2} s^{-1} and a photoperiod of 16 hrs.

Per each genotype, data on budbreaking and on the number of days required for sprouting were recorded for 4 weeks and the percentage of responding buds were calculated for both explanting times (September and December). After four months of culture, per each sprouted bud, the length of the main shoot, the number and the length of the lateral shoots and the multiplication rate (the number of new shoots obtained from the bud placed in culture) were recorded per two subcultures. Differences between the two explanting times, the two genotypes and the two subcultures were determined using analysis of variance (ANOVA). The variation among genotype means was analyzed using Tukey's Test and t-test.

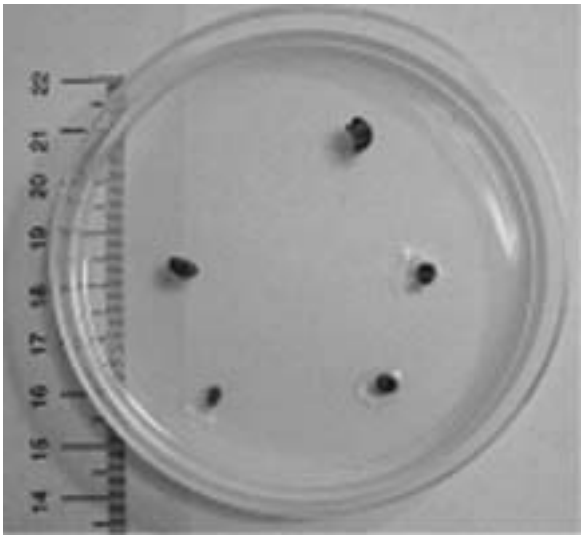

Fig. 1 — Five buds of FB in a Petri dish.

RESULTS AND DISCUSSION

Axillary buds started to break after one week of culture and kept on sprouting for 4 weeks (Fig. 2). In the first time of explanting (September), FN showed a higher percentage of sprouted buds (87.9%) than FB (85.5%). In the second time of culture (December), the percentage of open buds of FB was higher (58.3%) than of FN (40%). Statistical differences were observed between the two explanting seasons, but non between the two genotypes, showing a high seasonal effect on *in vitro* mulberry budbreaking (tab. 1). QURAISHI *et al.* (1996) suggested that differences in the physiological condition of the stock plants grown under natural environmental conditions might be the reason for differential growth responses *in*

Fig. 2 — Sprouted buds of FN.

Table 1 — Percentage of budbreaking of two genotypes after 4 weeks of *in vitro* culture

Genotype \ Explanting time	September	October
FN	87.9±7.78	40.0±10.70
FB	85.4±9.09	58.33±9.09
Genotype[2]	0.407	
Subculture[2]	<0.01	
Genotype X Explanting time[2]	0.463	

[2] P values for main factors and interactions of two-way ANOVA (Tukey's test).

vitro. In previous reports, summer is indicated as the best season for the plant material collection (BHAU and WAKHLU 2003; VIJAYA CHITRA and PADMAJA 2002).

Sprouted buds were transferred from Petri dish to test tubes. During the first subculture, new secondary shoots were produced (Fig. 3); so that it was possible to distinguish a main shoot (Fig. 4) and several lateral shoots (Fig. 5). Main shoots were statistically longer in FN than in FB (respectively 1.9 and 0.8 cm), independently on the number of subcultures. The same result was observed regarding the length of lateral shoots; in fact, the lateral shoots of FN were statistically longer than the ones of FB (respectively 0.7 and 0.5 cm). On the contrary, differences statistically not significative were observed between the genotypes regarding the number of lateral shoots (3.6 for FN and 4.2 for FB). Statistical differences were recorded between the subcultures; in fact, for both genotypes, the number of lateral shoots decreased, after the first subculture (tab. 2). There is a disagreement between our observations and

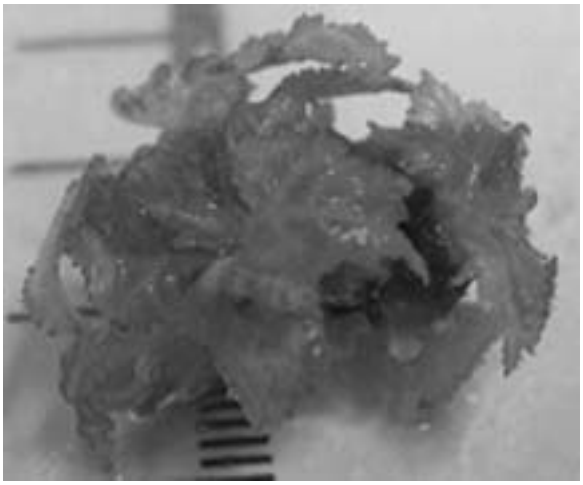

Fig. 3 — FN *in vitro* developed shoots.

Fig. 5 — FB lateral shoots.

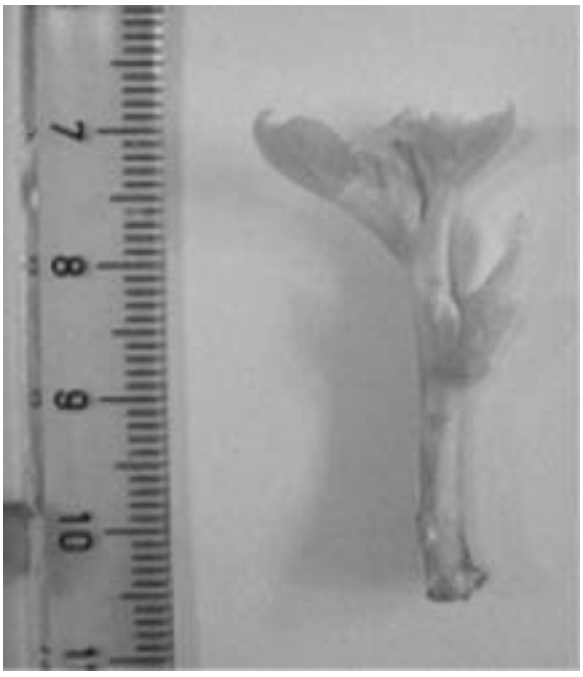

Fig. 4 — A FN main shoot.

Fig. 6 — Rooted FB shoot.

Table 2 — Characterization of shoots obtained after 4 months of *in vitro* culture

Genotype	Subculture	Main shoot length (cm)	Lateral shoot length (cm)	N° of later shoots	Multiplication rate[1]
FN	I	1.2±0.09	0.7±0.04	4.2±0.40	23.7±8.03
	II	1.8±0.13	0.8±0.04	3.0±0.41	
FB	I	0.9±0.22	0.5±0.03	4.9±0.41	28.3±4.26
	II	0.6±0.22	0.5±0.04	3.5±0.49	
Genotype[2]		<0.001	<0.001	0.043	0.027
Subculture[2]		0.381	0.294	0.03	-
Genotype X Subculture[2]		0.734	0.331	0.542	-

[1] Multiplication rate was calculated after 4 months of culture and data analyzed by t-test.
[2] P values for main factors and interactions of two-way ANOVA (Tukey's test).

the results reported for several mulberry genotypes by VIJAYA CHITRA and PADMAJA (2002) and BHAU and WAKHLU (2003).

After four months of culture, no differences were observed between the genotypes regarding the multiplication rate. In fact, per each bud it was possible to obtain 23.7 new shoots in FN and 28.3 new shoots in FB.

Root induction was obtained from proliferated shoots and no differences in the percentage of rooting were registered between the two genotypes (Fig. 6).

CONCLUSIONS

An efficient *in vitro* establishment system was set up for two Sicilian *Morus* genotypes, using axillary buds explanted in two different seasons from mature trees in open field. As previously reported by many authors, the response to *in vitro* culture was highly genotype and season dependent (TEWARY *et al.* 1995; PATTNAIK and CHAND 1997; LU 2002; BHAU and WAKHLU 2003). Using this protocol a high multiplication rate has been obtained after four months of culture for both genotypes.

Further studies are in progress to use the aseptic mulberry material obtained to achieve *in vitro* regeneration through organogenesis and embryogenesis, also aimed to establish effective protocols for *ex situ* germplasm preservation.

Aknowledgments — This work was supported by the National Project PRIN 2004 "In vitro morphogenesis and synthetic seed as innovative tool to propagate and to conserve the Mediterranean woody plant germplasm" (prot. 2004070973) (Responsible Maria Antonietta Germanà).

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Received November 30th 2006; Accepted January 30th 2007