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In vitro storage of plum germplasm by slow growth

S. Gianní, F. Sottile

Department of Agricultural and Forest Sciences, University of Palermo, Palermo, Italy

Abstract

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In this study, *in vitro* slow growth storage was investigated in four cultivars of two Sicilian (Southern Italy) plum species (*Prunus domestica* L. and *Prunus cerasifera* Ehrh. – two genotypes each). Established shoot cultures were preserved at 4°C in the dark in a Murashige and Skoog basal medium containing one of two different concentrations of sucrose (20 and 30 g/l) and with or without growth regulators. We tested the effects of cold storage, genotype and media on survival and re-growth capacity of explants after 3, 6, 9 and 12 months of storage. Effective minimal growth under cold conditions occurred in all four genotypes. The media composition did not affect survival, which, instead, appeared to be genotype-dependent. *P. domestica* genotypes survived cold storage the longest, for 12 and 9 months; instead, *P. cerasifera* ones remained viable for up to 6 months. All genotypes retained proliferation capacity under standard conditions and their re-growth capacity seems to be strongly genotype-dependent, closely related to their individual performance in response to the experimental condition of storage.

Keywords: genotype; *in vitro* preservation; plant genetic resources; Prunus

Plant genetic resources are the basis of future agriculture. They include traditional and modern cultivars as well as their wild relatives (Rao 2004). Human activities, such as urbanization and monoculture, contribute to the loss of diversity, including these natural sources of crop biodiversity. Many international projects have been founded to preserve plant genetic resources. The maintenance of a germplasm collection by *in vitro* techniques has the advantages of requiring less space and limited labour costs (Towill 1988).

Several *in vitro* methods have been developed for the genetic conservation of vegetatively propagated crops (Withers 1980). Among them, slow growth *in vitro* has been carried out in a wide range of species, and the duration of a subculture cycle can be extended from a few weeks to 6–12 or more months in some species. Advantages are that contaminations and genetic modifications are reduced (Reed et al. 1998). Slow growth can be achieved through several methods, such as using low temperature, darkness, low-light intensity, modification of minerals in the culture medium and use of osmotic agents and growth retardants. For cold-tolerant species, temperatures in the range of 0–5°C are employed, while for tropical species, which are usually cold sensitive, temperatures between 15–20°C are used (Engelmann 2011).

The temperate genus *Prunus* (family Rosaceae) comprises more than 200 species (Bailey, Bailey 1976) and includes fruit and ornamental species. *Prunus domestica* L. consists of hundreds of selected cultivars and includes most edible plums. Another important species is *Prunus cerasifera* Ehrh., in the myrobalan (or cherry plum) group used as ornamental tree for its foliage and flowering traits,

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as rootstock for selected genotypes of both European and Japanese plums and in some genotypes also for production of edible fruit.

In the present study, the *in vitro* preservation of four Sicilian plum genotypes belonging to *P. domestica* and *P. cerasifera* (two genotypes each) was conducted for the first time. These autochthonous genotypes have been widely cultivated in the past but are now at risk of loss, although they are particularly appreciated by consumers for their organoleptic qualities. Thus, our study could have a positive economical outcome by promoting germplasm exchange and rapid propagation when necessary.

We tested the effects of genotype, the composition of media and the experimental conditions of storage on survival and the potential re-growth after two consecutive subcultures under standard temperature and photoperiod. To our knowledge, little information exists on preserving *P. domestica* and especially *P. cerasifera* by slow growth, although some species and clones belonging to *Prunus* have been investigated by this technique (Marino et al. 1985; Sauer 1985; Janeiro et al. 1995; Pérez-Tornero et al. 1999; Lambardi et al. 2006). To slow down the growth, we preserved the genotypes at 4°C in the dark, as successfully tested in *Prunus* and in several other species (Marino et al. 1985; Sauer 1985; Orlikowska 1992; Hausman et al. 1994; Negri et al. 2000; Lambardi et al. 2006) testing two concentrations of sucrose and the presence/absence of plant growth regulators in the storage medium.

MATERIAL AND METHODS

Plant material and shoot proliferation cultures. Shoot cultures of four *Prunus* genotypes were initiated from young branches collected in the spring season from an *on-site* collection. The plants were *P. domestica* genotypes Sanacore and Ariddu di Core and *P. cerasifera* genotypes Marabolo and Rapparino. The shoots were defoliated, cut into segments with 3–4 nodes each, washed for 10 min in running tap water, washed in double-distilled water with 0.1% (v/v) Tween 20™ for 10 min, then rinsed three times in double-distilled water. Cuttings were sterilized by immersing in 2% (v/v) Plant Preservative Mixture™ (Plant Cell Technology, Inc, Jefferson Place, USA) for 20 min, in 70% ethanol for 3 min, then in 70% solution of commercial bleach (3.85% final sodium hypochlorite concentration) for 20 minutes. Finally, the segments were rinsed three times in sterile distilled water.

After sterilization, 2 cm long segments with one node in the middle were prepared. Explants were cultured until sprouting on initial culture medium consisting of MS basal medium with vitamins (Murashige, Skoog 1962), 30 g/l sucrose, 6 g/l agar (Plant Agar; Duchefa Biochemie B.V., Haarlem, The Netherlands), and 0.2% (v/v) PPM™. The medium was supplemented with plant growth regulators (PGRs): 6.65μM 6-benzylaminopurine (BAP) and 0.49μM indolebutyric acid (IBA); the pH was adjusted to 5.7 before autoclaving at 121°C for 20 minutes. Cultures were maintained at 24 ± 2°C, under a 16/8 h (light/dark) photoperiod and a light intensity of 50 μmol/m^2·s. After sprouting, 10–12 day old shoots were transferred to the same medium but with a reduced concentration of BAP (3.8μM) and cultured for 1 month. After that, cultures were subcultured monthly in Eco2™ Box (Eco2 NV, Ophasselt, Belgium) vessels with 60 ml of MS medium containing 30 g/l sucrose, 8 g/l agar, 0.2% (v/v) PPM™, 2.2μM BAP and 0.49μM IBA. When the cultures were well established and immediately prior to starting the cold storage experiment, we recorded the proliferation rate (mean number of proliferated shoots per explant) and the mean length (cm) for each genotype to serve as controls under standard conditions.

Minimal growth storage and repropagation. The slow-growth experiment was performed with 1.5–2 cm long shoots taken from established cultures. Explants were stored in an Eco2™ Box with 60 ml of medium. Four different media containing Murashige-Skoog medium with inorganic and organic nutrients, 8 g/l agar, and 0.2% (v/v) PPM™ at pH 5.7 were tested; they differed in the amount of sucrose and the presence/absence of plant growth regulators (PGRs) (Table 1). Each treatment for each genotype was performed with four replicates of four explants each (4 genotypes × 4 media × 4 replicates × 4 plants per replicate = 256 plants). Explants were placed in darkness in a cold room at 4°C and their viability was examined after 3, 6, 9, and 12 months. The survival data were taken at the end of the experiment as a percentage of vital stored shoots. At intervals of 3 months, stored explants were also visually observed to check their qualitative appearance, e.g., discoloration, brown-

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Table 1. Sucrose, BAP (6-benzylaminopurine) and IBA (indole-3-butyric acid) concentrations in the Murashige-Skoog-based media used during cold preservation

Treatment	Sucrose (g/l)	BAP (μM)	IBA (μM)
A	20	2.2	0.49
B	20	0	0
C	30	2.2	0.49
D	30	0	0

ing, necrosis, hyperhydricity, presence/absence of rooting, and effective minimal growth.

After each storage period, one replicate of cold-stored shoots per medium and genotype (4 genotypes × 4 media = 16 plants) was subcultured for two consecutive 30-day cycles at standard conditions. After the first subculture, cultured shoots (at least 0.5 cm in length) were placed in fresh medium for the second one. The number of shoots per explant and their lengths were recorded at the end of each subculture.

Statistical analyses. The experimental design was completely randomized. Survival values (%) were arcsin-square root transformed prior to a two-way analysis of variance (ANOVA) followed by the Tukey's honestly significantly different (HSD) test for $P < 0.05$. The effects of 'genotype', 'treatment' and their interaction (genotype × treatment) were statistically analysed. Proliferation rate and the length of shoots were statistically examined for each genotype. Count data for the number of proliferated shoots were analysed after decimal log(X + 10) transformation. All the multiple comparisons were performed using the one-way ANOVA ('treatment' or 'storage period') followed by the Tukey's HSD or Scheffe's tests to distinguish mean differences while mean differences between samples were tested by the Student's *t*-test. Data for the length of proliferated shoots were analysed by the Kruskal-Wallis non-parametric one-way ANOVA by ranks and the non-parametric multiple test for all-pairs comparisons with Bonferroni correction described by Gao et al. (2008) was applied. To non-parametrically compare two samples, we used the Wilcoxon rank-sum test. All statistical analyses were carried out using the R environment (R Core Team 2013).

RESULTS AND DISCUSSION

In this study, plum genotypes clearly behaved differently during cold storage at 4°C and darkness (Fig. 1). Among genotypes, survival rate varied significantly. Statistically, results indicated that 'genotype' had a highly significant effect on survival ($P < 0.0001$), unlike 'treatment' ($P = 0.879$). The interaction genotype × treatment was not significant. In the post-hoc analysis, *P. domestica* genotypes were significantly different from the *P. cerasifera* ones. *Prunus domestica* genotypes also exhibited statistically-significant differences from one another, while the *P. cerasifera* genotypes survived at statistically similar rates (Table 2). Species and variety respond differently to cold storage (Monette 1988; Wilkins et al. 1988; Reed et al. 1998; Kovalchuk et al. 2009). The *P. cerasifera* and *P. domestica* genotypes adapted well to *in vitro* culture until the establishment and rooting phase. However, some genotypes produced fewer viable shoots under cold conditions. We saw

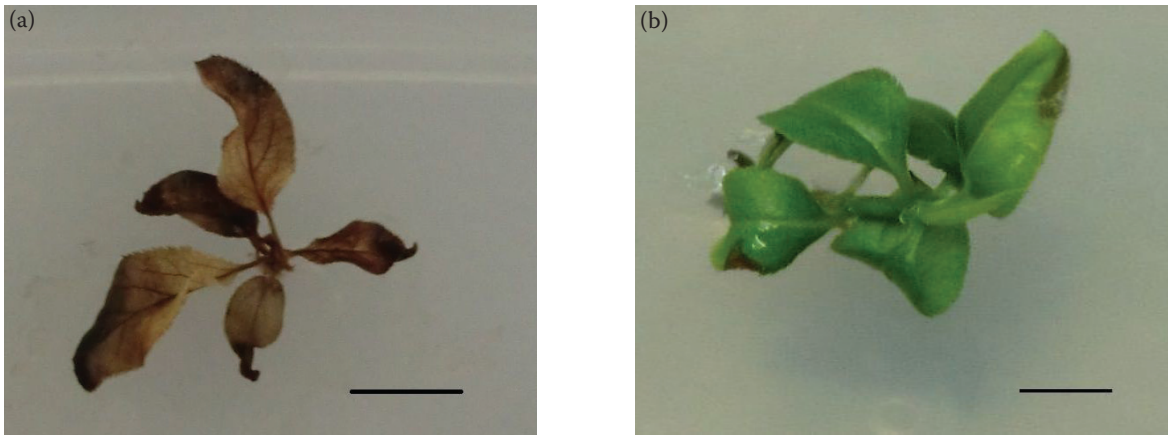

Fig. 1. Cold-stored explants. (a) necrotic shoot in cv. Marabolo (bar 10.0 mm) and (b) vital explant in cv. Ariddu di Core (bar 10.0 mm)

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Table 2. Survival (%) of plum shoots recorded at the end of cold storage periods at 4°C in darkness

Genotype	Treatment			
	A	B	C	D
Ariddu di Core	100^{aA}	94^{aA}	94^{aA}	94^{aA}
Sanacore	75^{bB}	75^{bB}	75^{bB}	75^{bB}
Marabolo	25^{cC}	44^{cC}	44^{cC}	31^{cC}
Rapparino	25^{cC}	44^{cC}	50^{cC}	44^{cC}

values sharing the same letter do not differ statistically at $P \leq 0.05$ according to the Tukey's (HSD) test. Different capital letters in the same row indicate significant differences among treatments; different lowercase letters in the same column indicate significant differences among genotypes

distinct differences between both species and within *P. domestica* during cold storage. Thus, the adaptability of *in vitro* cultures to cold storage could be genotype-dependent, and survival might be genetically determined. These results agreed with the observations of Wilkins et al. (1988) and Moriguchi et al. (1990), who reported that survival rates during *in vitro* preservation were variety-dependent in apple and among European and Japanese pear varieties, respectively. Cvs Ariddu di Core and Sanacore remained viable for 12 and 9 months, respectively; thus they seem to be more cold tolerant than cvs Marabolo and Rapparino that survived for 6 months as reported in Table 2 in which all the varieties show marked differences in survival percentages. In addition, in Tables 3–4 the missing values indicate died shoots during storage or no regeneration during the regrowth. Cold storage resulted in necrosis and browning of explants that usually started in the apical region and spread basally over time. Cv. Marabolo was more rapidly affected by browning than cv. Rapparino, which was affected during the second 3 months of storage; both cultivars showed the lowest survival to cold temperature. Instead, cv. Ariddu di Core was slightly influenced at 12 months, while cv. Sanacore showed few necrotic symptoms between 6 and 9 months. With respect to survival, we did not find significant differences among treatments within the same cultivar during cold storage.

Cold is the simplest and most efficient method to slow *in vitro* growth (Druart 1985). According to several studies, *in vitro* slow growth storage works also best in darkness in many species, such as *Prunus* (Marino et al. 1985), *Malus* (Orlikowska 1992), *Populus* (Hausman et al. 1994), and *Quercus suber* (Romano, Martins-Loução 1999). Adaptation to cold and to the presence or absence of light is genotype-dependent at both the interspecific and intraspecific levels and can affect the appearance of cultures and the duration of storage by months or years (Reed 1998). The absence of proliferation and rooting during our cold storage experiment demonstrated that slow growth preservation was accomplished. The limited growth of the *in vitro* cultures (we observed only a very slight increase in shoot length) resulted from slowed cellular metabolism (Engelmann 2011), while the apical etiolation could be caused by darkness. We did not observe hyperhydric shoots during storage.

During the storage, we tested different compositions of media (20 and 30 g/l sucrose, with and without PGRs). We observed the presence of senescent basal leaves only during cold storage and mainly in treatment D (without PGRs), but to a lesser extent in treatment C. Leaves in treatments A and B (20 g/l sucrose) were green and less senescent but prone to weakness and fell off the plant easily, even though explants remained viable. Pruski et al. (2000) found also yellow basal leaves in *Prunus virginiana* L. under cold storage both in dark and light conditions ascribing it to the presence of sucrose in the storage media. The presence of exogenous sugars is thought to limit the photosynthesis by several works in many species (Hdider, Desjardins 1994; Van Huylenbroech, Debergh 1996; Tichá et al. 1998; Rybczynski et al. 2007; Yuanyuan et al. 2009) according to Koch's theory (Koch 1996) on the inhibitory influence of sugars on photosynthesis. We detected that the presence of PGRs seems to improve the quality of leaves. In addition, during the regrowth phase at standard conditions of media (30 g/l of sucrose and PGRs) and light, stored explants regenerated without senescent leaves similarly to non-cold-stored cultures. Contrary to what previous studies commonly considered (e.g. Wittenbach 1977; Biswal, Biswal 1984; Veierskov 1987), the study

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Table 3. Mean number of shoots per explant detected for each genotype after the first and the second subcultures under standard conditions after each cold storage period

Genotype	treatment	1st Subculture				2nd Subculture			
		months				months			
		3	6	9	12	3	6	9	12
Ariddu di Core	A	3.75	4.00	4.75	$2.00^{n.s.}$	5.00^{A}	5.00^{A}	4.25^{AB}	2.83^{B}
	B	3.50	3.67	2.33	$2.00^{n.s.}$	4.17	3.25	3.50	$2.00^{n.s.}$
	C	3.75	3.25	5.25	$2.67^{n.s.}$	4.20	3.25	4.38	$4.33^{n.s.}$
	D	3.50	3.50	2.50	$2.33^{n.s.}$	4.86	4.50	2.50	$2.33^{n.s.}$
	control	4.90	4.90	4.90	4.90	4.90	4.90	4.90	4.90
		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Sanacore	A	5.50	6.75	4.50	– n.s.	5.43	5.20	4.75	– n.s.
	B	6.33	3.25	3.50	– n.s.	4.55	3.33	4.25	– n.s.
	C	3.25	4.75	5.50	– n.s.	4.89	4.25	6.67	– n.s.
	D	4.33	4.50	5.50	– n.s.	4.22	3.30	4.43	– n.s.
	control	5.26	5.26	5.26		5.26	5.26	5.26	
		n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	
Marabolo	A	4.33	–	–	–	2.80^{a}	–	–	–
	B	3.50	2.00^{a}	–	– n.s.	3.80^{ab}	3.00^{a}	–	– n.s.
	C	4.75	3.67^{ab}	–	– n.s.	3.00^{abA}	1.33^{aB}	–	–
	D	3.33	–	–	–	3.25^{ab}	–	–	–
	control	5.05	5.05^{b}			5.05^{b}	5.05^{b}		
		n.s.							
Rapparino	A	3.00	–	–	–	4.50	–	–	–
	B	2.25	1.50^{a}	–	– n.s.	3.80	2.00	–	– n.s.
	C	5.75^{A}	3.50^{abB}	–	–	5.50	3.90	–	– n.s.
	D	2.33	–	–	–	2.00	–	–	–
	control	4.50	4.50^{b}			4.50	4.50		
		n.s.				n.s.	n.s.		

means sharing the same lowercase letter(s) within a column were not significantly different at $P \leq 0.05$ according to the Tukey's (HSD) or Scheffe's test; means sharing the same capital letter(s) within a row were not significantly different at $P \leq 0.05$. Among periods of storage, the multiple comparisons were performed using the Tukey's HSD or Scheffe's test while mean differences between samples were tested by the Student's *t*-test; n.s. – no significant differences; (–) – no regeneration or died during storage

of Weaver and Amasino (2001) proved in *Arabidopsis thaliana* that senescence is not induced by darkness as well as it is not inhibited by light. In our study, senescent leaves were observed as more evident in cvs Ariddu di Core and Sanacore than in the other species. This observation could also suggest a different genotypic response to the experimental conditions. We also observed that in cvs Marabolo and Rapparino explants went directly to necrosis and browning without turning yellow before. Unlike necrosis and browning that affected the whole explants, yellowish leaves did not compromise survival and re-growth capacity. In our investigation, 2.2μM cytokinin could have been sufficiently low to attenuate senescence, as reported by Wilkins et al. (1988) and Ahmed et al. (2011).

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Table 4. Mean length of proliferated shoots detected for each genotype after the first and the second subcultures under standard conditions after each cold storage period

Genotype	Treatment	1st Subculture				2nd Subculture			
		months				months			
		3	6	9	12	3	6	9	12
Ariddu di Core	A	0.29^{aA}	0.39^{AB}	0.51^{B}	0.58^{B}	0.68^{AB}	0.90^{abA}	0.35^{abB}	0.45^{AB}
	B	0.61^{ab}	0.44	0.49	$0.43^{n.s.}$	0.68^{A}	0.85^{abA}	0.31^{aB}	0.33^{B}
	C	0.71^{bc}	0.54	0.62	$0.81^{n.s.}$	0.60^{AB}	0.87^{aA}	0.63^{bAB}	0.42^{B}
	D	0.94^{b}	0.54	0.56	$0.51^{n.s.}$	0.65^{AB}	0.79^{abA}	0.51^{abAB}	0.37^{B}
	control	0.50^{ac}	0.50	0.50	0.50	0.50	0.50^{b}	0.50^{ab}	0.50
			n.s.	n.s.	n.s.	n.s.			n.s.
Sanacore	A	0.64	0.53	0.59	– n.s.	0.65^{A}	0.89^{B}	0.64^{AB}	–
	B	0.63	0.65	0.54	– n.s.	0.69	0.79	0.66	– n.s.
	C	0.67	0.81	0.45	– n.s.	0.69	0.79	0.45	– n.s.
	D	0.69	0.72	0.56	– n.s.	0.79^{A}	0.82^{A}	0.50^{B}	–
	control	0.70	0.70	0.70		0.70	0.70	0.70	
		n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	
Marabolo	A	0.29^{a}	–	–	–	0.79	–	–	–
	B	0.68^{ab}	1.55	–	– n.s.	1.21^{A}	0.86^{B}	–	–
	C	0.74^{ab}	0.79	–	– n.s.	1.31	0.75	–	– n.s.
	D	0.67^{ab}	–	–	–	1.08	–	–	–
	control	0.76^{b}	0.76			0.76	0.76		
			n.s.			n.s.	n.s.		
Rapparino	A	0.52	–	–	–	0.96	–	–	–
	B	0.47	0.93	–	– n.s.	1.01^{A}	0.52^{B}	–	–
	C	0.71	1.01	–	– n.s.	1.14	0.95	–	– n.s.
	D	0.40	–	–	–	0.73	–	–	–
	control	0.62	0.62			0.62	0.62		
		n.s.	n.s.			n.s.	n.s.		

means sharing the same lowercase letter(s) within a column were not significantly different at $P \leq 0.05$; n.s. – no significant differences; , means sharing the same capital letter(s) within a row were not significantly different at $P \leq 0.05$. The Kruskal–Wallis non-parametric one-way ANOVA by ranks and the non-parametric multiple test for all-pairs comparisons with Bonferroni correction described by GAO et al. (2008) were applied. To non-parametrically compare two samples, the Wilcoxon rank-sum test was used. Missing values indicate no regeneration or died during storage

Several authors ascribed necrosis and browning during cold storage to the presence of light and found them to be less pronounced in darkness (Hausman et al. 1994; Romano, Martins-Loução 1999) or when BAP was lacking in the storage medium (Orlikowska 1992). Furthermore, Bairu et al. (2009) supposed that when plants are cultured in a cytokinin-free medium, cytokinins are depleted from the shoots; the absence of roots decreases the endogenous cytokinin level, stopping cell division and causing apical necrosis. However, this interpretation may not explain our cold chamber results. In fact, necrosis appeared irrespective of the media (with or without cytokinin) but was clearly genotype-dependent, maybe a signal of a different cold sensitivity. Thus, the lowest survival percentages (about 25–31%) oc-

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curred in the *P. cerasifera* cultivars. The absence of treatment effects on survival could suggest that genotype had the greatest influence.

We analysed the effects of 'treatment' and 'storage period' separately by one-way ANOVA after stored cultures had been transferred to fresh medium (Fig. 2). Data were analysed for the first and the second subculture at 3 month intervals by both proliferation rate (Table 3) and mean length of proliferated shoots (Table 4). We assessed (1) which treatment had the best results compared with the control and other media over the same period of storage and (2) the length of storage after which cultures could regenerate without a significant decline in the proliferation rate and length of shoots. The results suggest that genotype-affected behaviour when cold-stored cultures were replaced into the multiplication medium under standard conditions. Cvs Ariddu di Core and Sanacore did not show significant differences among treatments as proliferation rate during either subculture cycles, but we observed significant differences in the two *P. cerasifera* genotypes. Specifically, both genotypes proliferated during the first 90 days of subculture similarly to the control, but after the second 3-month interval, cold-stored explants in treatment B performed at lower rates than the control in cvs Marabolo (P = 0.0028) and Rapparino (P = 0.019); additionally, explants in treatments A and D could not regenerate, although they had only slight necrosis. In the second subculture, cv. Rapparino regenerated without significant differences

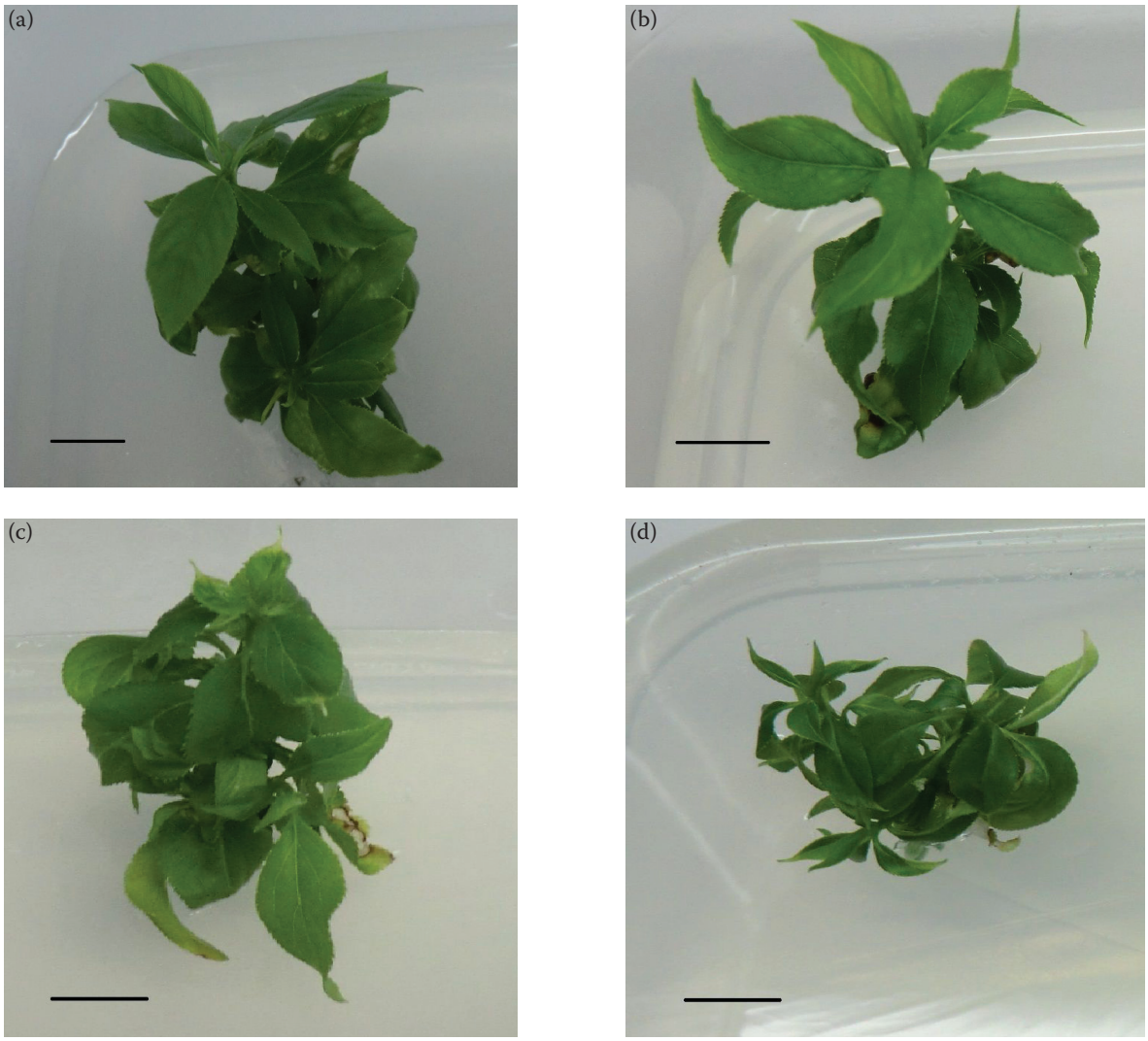

Fig. 2. Regenerating plantlets of plum evaluated after the first subculture under standard medium, temperature, and light conditions after cold storage (bar 10.0 mm). Genotypes: (a) Ariddu di Core, (b) Marabolo, (c) Rapparino and (d) Sanacore examples were cold preserved in the Murashige-Skoog medium supplemented with 30 g/l of sucrose, 2.2μM BAP, and 0.49μM IBA

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among treatments compared also with the control, but cv. Marabolo re-grew significantly less than the control in treatment A at 90 days (P = 0.044) and in treatments B and C at 6 months (P = 0.031 and P = 0.002, respectively). With respect to storage period, some genotypes showed largely stable results. Overall, most of the viable stored cultures retained re-growth capacity, in many cases reaching or exceeding the level of the control. The inability to regenerate after 6 months of culture in treatments A and D of cvs Marabolo and Rapparino could be because they were sensitive to the experimental conditions. However, the same treatments regenerated in the other species, indicating that genotype was more important than treatment during regeneration as well. Thus, all treatments compared with the control showed the effects of genotype in the number and length of proliferated shoots, probably because of different degrees of cold sensitivity.

We also analysed differences in each treatment from 90 days to the last monitoring period (180, 270, or 360 days). The statistically significant differences, when found, were mainly associated with the reduced proliferation rate and shoot length only in the last storage period. Additionally, we observed that most comparisons were not significantly different in the first subculture, which offers important information about genotype's re-growth ability immediately after cold storage. Several authors have assumed that prolonged cold exposure could reduce dormancy and induce vigorous plantlets (Mullin, Schlegel 1976; Pérez-Tornero et al. 1999), benefitting re-growth. Our results for the first subculture largely supported these assertions. We also compared both cycles at the same storage period (data not tabulated). Results proved that all varieties largely preserved their re-growth capacities in the second subculture; when significant differences from the first one were found, they were mainly in greater shoot elongation in the second cycle. The proliferation rate was largely consistent in both cycles.

In conclusion, our study showed that the results were strongly affected by genotype. Interestingly, we observed a clearly different response between species and among genotypes. Storage at 4°C in darkness was effective for genotypes like cv. Ariddu di Core and Sanacore but less so for the cv. Marabolo and Rapparino varieties. We observed that the addition of PGRs improved the quality of the cold-stored material. Our results from storage at 4°C were rather encouraging; therefore, cvs Marabolo and Rapparino may require a warmer storage temperature (about 8–10°C), although effective slow growth at temperatures above 4°C must be checked.

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Corresponding author:

Prof. Francesco Sottile, University of Palermo, Department of Agricultural and Forest Sciences,
Viale delle Scienze 11, 90128 Palermo, Italy
phone: + 39 091 238 61200, fax: + 39 091 651 5531, e-mail: francesco.sottile@unipa.it