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Proceeding Paper

Establishment and Optimization of Micrografting Assays with Almond (*Prunus dulcis*) Portuguese Varieties †

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Abstract: In the last years almond culture has increased in Portugal with an introduction of new orchards neglecting the traditional varieties. Micrografting, grafting in in vitro conditions, is a technique that has been established for commercial almond trees, but no studies have been described with Portuguese varieties. In this work, an efficient protocol for almond micrografting with traditional almond trees was established. The effect of plant growth regulators (BAP and IBA) and activated charcoal on culture medium were also evaluated during micrografting assays. Besides that, the effect of auxin IBA on root induction was analysed during rooting assays.

Keywords: Almond tree; micrografting; growth regulators

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1. Introduction

Almonds (*Prunus dulcis*, (Mill.) D.A. Webb) are an important tree nut crop cultivated worldwide [1]. Portugal has a long history of almond production in Algarve, Trás-os-Montes and Douro, but in recent years the almond growing area has increased in the Alentejo and Beira Interior regions, mainly due to water availability. However, the majority of the new plantations have used foreign cultivars, neglecting the traditional varieties, known for the high quality of the fruits [2].

Micrografting is a technique developed in the 80s that consists in the placement of the scion onto a decapitated rootstock in aseptic conditions [3]. This technique is used for elimination of virus [4,5], to determine the compatibility between the scion and the rootstock [6], rejuvenation of mature tissues [7,8], and large-scale production of disease-free plants [9].

Micrografting is divided into three main stages: establishment and multiplication of scions (i), establishment and multiplication of rootstocks (ii) and preparation of the rootstock and scion for micrografting (iii) [9]. The compatibility between the scion and rootstock, the culture medium, the grafting type, the endogenous organic compounds and growth regulators are known to influence the growth rate and success of the micrograft [10].

Some protocols have been described for commercial almond trees [3,11,12] but no one has been established for traditional varieties. Therefore, in this work, *slit micrografting* technique was evaluated in different traditional Portuguese varieties (Rabo de Zorra, Gama Dura and Canhota). The effect of the plant growth regulators (PGR), 6-

Benzylaminopurine (BAP) and indole-3-butyric acid (IBA), and of activated charcoal (AC) on culture medium were also analyzed during micrografting assays, in order to establish a protocol for micrografting with almond Portuguese varieties.

2. Materials and Methods

2.1. Establishment of In Vitro Cultures for Scion and Rootstock

Mature kernels from Rabo de Zorra, Gama Dura and Canhota varieties (scions), and bitter almond (rootstock) without the shells, were surface sterilized by immersion in water with detergent, immersion in a solution of fungicide (Derosal 1 g/L) for 20 min, immersion in a commercial bleach solution with 3.75% active chlorine for 20 min, and washed three times with sterile water in flow chamber. After sterilization, the seed coats were removed and the seeds were placed in a petri dish lined with sterile paper moistened with a 1 mg/L gibberellic acid (GA_3) solution. The petri dishes were incubated in a growth chamber at 25 ± 1 °C under a 16-h photoperiod. After germination, in vitro seedlings were decapitated above the cotyledons and cultured in MS (Murashige and Skoog) medium including 30 g/L sucrose, 1 mg/L BAP and 7 g/L agar. The cultures were placed and maintained for 3 weeks, in a growth chamber with the same conditions described before, and subcultured every 3 weeks to the same medium.

2.2. Rooting Assays

Explants from Canhota and bitter almond were dippe 30 minutes in a solution of 1 g/L IBA and transferred to MS medium including 30 g/L sucrose and 7 g/L agar. The cultures were placed and maintained in a growth chamber in the same conditions described before. The rooting rate and the total number of roots was registered after 2 months.

2.3. Micrografting Assays

2.3.1. Effect of the Final Medium in Micrografting

The slit micrografting that consists in a vertical slit on the rootstock and a cut in a v-shape on the scion base was the method used in this work (Figure 1). The scion was then fitted in to the slit and the micrografts were cultured in three different media: M1) MS medium including 30 g/L sucrose and 7 g/L agar, M2) M1 medium supplemented with 1 mg/L BAP, M3) M1 medium supplemented with 2 g/L AC. Bitter almond × Canhota micrografts and homografts (R.Zorra × R.Zorra; Canhora × Canhota; G.Dura × G.Dura) combinations were tested.

The cultures were placed and maintained for 2 months in a growth chamber with the same conditions described before. Micrograft length (cm), healing rate (%), success of micrografts (%) and shoot formation in the rootstock (%) were registered after 2 months. The success of micrografts was recorded considering the healing without callus differentiations and scion displacement along with micrograft growth.

2.3.2. Rooting during Micrografting

By slit micrografting Rabo de Zorra, Gama Dura, Canhota (scions) and bitter almonds (rootstock) were cultured in three different conditions: (1) MS medium with 30 g/L sucrose and 7 g/L agar (control); (2) MS medium with 30 g/L sucrose, 1 g/L IBA and 7 g/L agar; (3) MS medium with 30 g/L sucrose and 7 g/L agar after rootstock dipping for 1 minute in a 1 g/L IBA solution. For each condition, 15 micrografts were used (5 for each variety). The cultures were placed and maintained for 2 months in a growth chamber with the same conditions described before. The same parameters described before were used in the evaluation of the micrograft assay.

2.4. Statistical Analysis

Statistical analysis was performed using STATISTICA 7.0. Kruskal-Wallis test at $p \leq 0.05$ was used to analyze micrograft length (cm), after 2 month of micrografting.

3. Results and Discussion

Scions and rootstocks were successfully in vitro established (Figure 1). After three multiplication cycles, the higher multiplication rate was obtained for bitter almond, when compared to the others varieties (data not shown). From scion varieties tested, G. Dura presented the higher multiplication rate (55%), in spite of no significant differences ($p \leq 0.05$) were obtained between scion varieties.

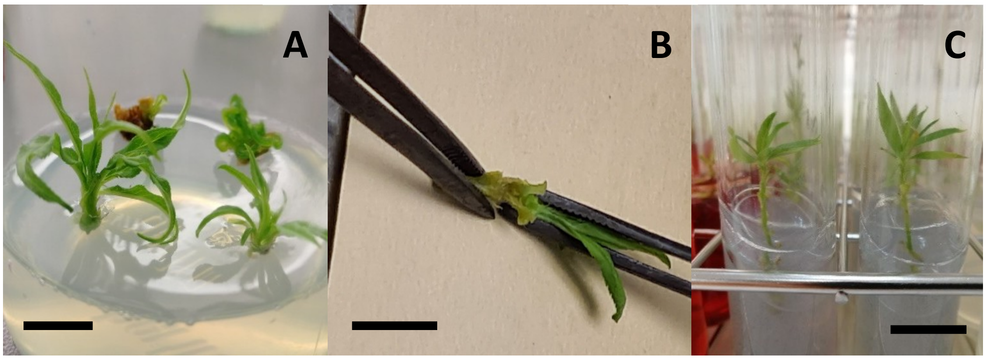

Figure 1. Micrografting of *Prunus dulcis*. Establishment and multiplication of rootstocks (**A**); *slit micrografting* (**B**); micrografts in MS medium (**C**). Scale bar—1cm. The micrografts were cultured in MS medium + 30 g/L sucrose + 7 g/L agar (M1 medium); M1 medium + 1 mg/L BAP; M1 medium + 2 g/L AC. The micrografting success were evaluated after 2 months.

3.1. Rooting Assay

Treatments with exogenous auxins such as IBA, α-naphthaleneacetic acid (NAA) or indole-2-acetic acid (IAA) demonstrated a beneficial effect in adventitious root induction of woody species [13]. In our study, the IBA dipping procedure seems to influence the formation of roots in bitter almond (60%), but not in the variety Canhota (6.6%), after two months of culture (Table 1). However, a high rate of callus formation was observed in bitter almond plants. Namli et al. (2011) evaluated in in vitro rooting of almond cultivar 'Nonpareil' using dipping treatment into 1.0 g/L of IBA at different durations (10, 15, 20, 25, 30 and 35 minutes), and the best root formation, 30%, was observed on the MS media (half strength) and dipped shoots 30 minutes at 1.0 g/L of IBA [14]. In contrast, Ainsley et al. (2001) tested different concentration of IBA over a range of incubations periods and obtained 60% rooting with "Nonpareil" and "Ne Plus Ultra" cultivars with shoot insertion for 12 h with 1.0 mM IBA, followed insertion in PGR free basal medium, but with 100mM phoroglucinol [13].

Table 1. Effect of IBA dipping procedure in the formation of roots; n = 15.

	Rooting Rate (%)	Total Number of Roots
Bitter almond	60	13
Canhota	6.6	1

3.2. Micrografts Assays

A good contact between the scion and rootstock, culture conditions and media have been crucial for successful graft unions [12]. Improvement of grafting by PGR exogenous application has been described by some authors [15]. However, in in vitro micrografting of cherry (Prunus avium), additional BAP did not improve grafting [16]. AC, frequently used in tissue culture due to its irreversible adsorption of inhibitory compounds (toxic metabolites, phenolic exudation and brown exudate), improves cell growth and development [17]. In the present assay, the use of BAP and AC in the final medium was studied in several scion × rootstock combinations. Similar healing rate mean were observed (87.1 to 90.9) in the three tested media. However, a decreasing success rate in relation to the healing rate was observed, mainly due to the formation of callus in the graft union and scion displacement. The use of PGR and AC free MS medium seems to influence the success of micrografting (72.72%) when compared to the MS+BAP (62.5%) and MS + AC (45.16%) media (Table 2).

Considering the different combinations of scion and rootstocks analysed, similar healing and success rates were obtained in the Bitter almond × Canhota micrograft, in the different culture media. These heterografts also presented a higher micrograft length mean, reinforcing the good compatibility observed between Bitter almond rootstock and Canhota variety.

Table 2. Effect of final medium in micrograft length, healing rate, success rate and shoot formation in the rootstock in micrografting.

			MS + BAP		
	N	**Micrograft Length (cm)**	**Healing (%)**	**Micrograft Success (%)**	**Shoot Formation in the Rootstock (%)**
Bitter almond × Canhota	4	2.75	100	100	100
R. Zorra × R. Zorra	5	1.42	80	40	40
		2.01 ± 1.2 a	88.9	62.5	66.67
			MS + AC		
	N	**Micrograft Length (cm)**	**Healing (%)**	**Micrograft Success (%)**	**Shoot Formation in the Rootstock (%)**
Bitter Almond × Canhota	8	3.3	75	75	75
R. Zorra × R. Zorra	6	2.08	100	33.33	83.3
Canhota × Canhota	7	1.98	100	85.7	57.14
G. Dura × G. Dura	10	1.75	100	30	30
		2.26 ± 1.5 a	87.1	45.16	58.06
			MS		
	N	**Micrograft Length (cm)**	**Healing (%)**	**Micrograft Success (%)**	**Shoot Formation in the Rootstock (%)**
Bitter Almond × Canhota	10	2.18	80	70	20
R. Zorra × R. Zorra	9	1.75	88.89	88.89	77.78
Canhota × Canhota	6	1.8	100	16.67	66.67
G. Dura × G. Dura	8	1.84	100	100	62.5
		1.91 ± 0.4 a	90.9	72.72	54.54

In a second assay, the induction of roots during micrografting was evaluated by IBA application. The use of IBA seems to improve the micrograft growth, the healing rate and the rooting rate. The micrograft growth was significantly higher in MS medium supplement with IBA, compared to PGR free MS medium ($p \leq 0.05$) (Figure 2a). However, the presence of callus in the micrografts treated with IBA (in the medium or by dipping process) may compromise the graft success during acclimatization (data not shown). IBA demonstrated a positive effect on root formation with 27% in MS medium supplement with IBA, 60% with the quick-dip approach (dipping) and only 13% in the MS medium (Figure 2b). In relation to the number of roots, the same effect was observed, with the formation of a higher number of roots with the quick-dip approach. Yıldırım et al. (2013) with different media (proliferation medium with 0.5 mg/L BAP and 0.1 mg/L IBA, rooting medium 0.5 mg/L IBA and 0.1 mg/L BAP and hormone-free medium) did not observe significant differences in micrografting success in "Texas", "Ferrastar" and "Nonpareil" cultivars, but observed a plant survival decrease during acclimatization when the micrografts were established in hormone-free MS medium, reinforcing the importance of PGR absorption from the medium in the survival of micrografts *ex vivo* conditions [11].

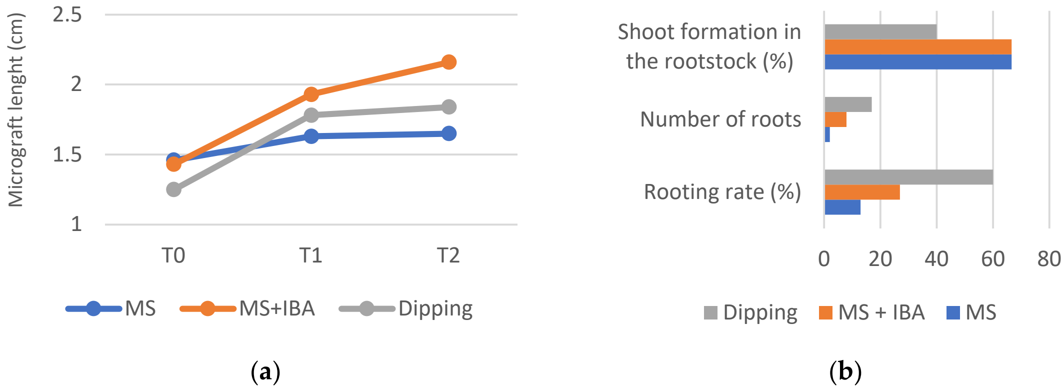

Figure 2. Effect of auxin IBA on micrografting. (**a**) Micrograft length evaluation after (T0), 1 month (T1) and 2 months (T2) of micrograft; (**b**) Shoot formation in the rootstock (%), number of roots and rooting rate (%), after 2 months of micrografts. Significantly differences were registered between MS and MS+IBA on micrograft length after 2 months (T2), at $p \leq 0.05$, using Kruskal-Wallis test.

4. Conclusions

Micrografting can be an alternative tool for vegetative propagation of fruit trees, when it's considered the health status and juvenility of the scion and rootstocks. Almond trees can suffer from viral diseases, such as Prune dwarf virus (PDV) and Prunus necrotic ringspot virus (PNRSV), which leads to great productivity losses [18,19]. To our knowledge this is the first report using traditional Portuguese varieties on micrografting. A success rate of micrografting around 73% was achieved with PGR free MS medium. IBA influences the root formation, and the dip-quick approach on rootstock contributes to the growth of the scions. However, due to the highest formation of callus that can influence the plant development during acclimatization, other concentrations and/or others PGR need to be studied in these varieties. This work represents a step forward in the field of multiplication of traditional almond varieties disease-free, which has been overlooked in the news almond orchards.

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