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Large-scale propagation of Myrobolan (*Prunus cerasifera)* in RITA® bioreactors and ISSR-based assessment of genetic conformity

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ABSTRACT

An efficient protocol for the micropropagation of *Prunus cerasifera* using temporary immersion bioreactors was described. One-node cuttings excised from field-grown plants were successfully disinfected using 12% sodium hypochlorite for 10 min. Immersion duration, frequency and medium composition in Bioreactors type RITA® were evaluated. A high multiplication rate was obtained using RITA® bioreactors containing Murashige and Skoog's medium (MS) supplemented with 1 mg L^{-1} N6-benzyladenine (BAP) and 1 mg L^{-1} indole-3-butyric acid (IBA) with 20 min/12 h as immersion time. RITA®-derived shoots were found to be more vigorous than those regenerated using the standard procedure. Higher levels of photosynthetic pigments were observed in shoots cultured in bioreactors, during the proliferation and rooting stages, which proved a certain degree of photoautotrophy of RITA®-derived vitroplants. A high proportion of shoots was successfully rooted in an MS liquid medium supplemented with 1 g L^{-1} activated charcoal in RITA® bioreactors. During plant acclimatization, survival rates exceeding 80% were recorded. The genetic fidelity of micropropagated plants was investigated using inter simple sequence repeat (ISSR) markers. Results proved the generation of homogenous amplification profiles and thus supported the clonal fidelity of regenerated vitroplants.

1. Introduction

Over the last decades, it has become necessary to create a number of easily propagated and adaptive rootstocks of stone-fruit crops to cope with environmental difficulties, notably drought, leading to the shortage of these varieties. The demand for elite *Prunus* rootstocks, for example, is increasing due to their recognized agronomical advantages, such as scion compatibility, nematode resistance against diseases (viruses, bacteria, fungi and insect) and edaphic adaptation (water-logging tolerance, salt, calcareous soils…). *Prunus* rootstocks play an important role in different breeding programs aimed at developing cultivars which are adapted to severe climatic conditions. These programs are based on interspecific crosses to broaden the genetic base, and thus allowing the introgression of new genes and conventional propagation. Moreover, breeding programs and micropropagation technologies have expanded improvement prospects for *Prunus* rootstocks; but the potential of these projects still requires a continuous improvement of rootstock characteristics. *Prunus cerasifera* (*P. cerasifera,* Myrobolan), one of the most abundant rootstocks in the Mediterranean area, belongs to the *Prunus* genus family tribe Amygdaleae and subfamily Spiraeoideae of the large Rosaceae family (Potter et al., 2007). Myrobolan has become economically important due to its great quality traits, which are based on its vigor (Salesses, 1975) and its resistance to root knot nematode (Meloidogyne spp.) (La Massese et al., 1984). This rootstock is usually propagated either by using vegetative methods (grafting or softwood cuttings) or through seed cultivation. Due to the low efficiency of such conventional methods together with the need for genetically homogeneous rootstock material, tissue culture technology has progressively been favored. In fact, Micropropagation enables the large-scale production of true-to-type and disease-free plants. It is also reliable for *in vitro* conservation of rare, endangered and threatened germplasm (Norton and Norton, 1986; Arbeloa et al., 2009; Debnath and Teixeira da Silva, 2007). Indeed, micropropagation techniques can improve the productivity of rootstock nurseries which may save up to a whole year in the production of a grafted tree when using tissue cultured material. Plant growth regulators (PGRs) are critical media components in determining developmental pathways of *in vitro* cultured explants. Among them, 6-benzylaminopurine (BAP) is commonly used to reduce the apical meristem dominance and induce both axillary and adventitious shoot formation

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from meristematic explants (Jafari et al., 2011). Micropropagation protocols using axillary buds are mainly adopted for the large-scale propagation of plants (Simmonds, 1983).

The use of simple bioreactors based on the temporary immersion concept can be considered as an effective innovation in plant tissue culture. The main advantage of this system lies in the fact that plant tissue can absorb nutrients directly from the medium (Paek et al., 2005). Periodic flows of sterile air enable a short contact of explants with liquid medium while renewing the vessel atmosphere. Multiplication using the Temporary Immersion System (TIS) yields an increase in biomass productivity. So, TIS could provide an adequate solution to the high cost of large scale propagation of rootstock using conventional tissue culture techniques (Niemenak et al., 2008; Zhu et al., 2005; Quiala et al., 2006). In fact, the multiplication rates for many species are considerably improved using such bioreactors (Takayama and Akita, 1998; Akdemir et al., 2014; Godoy et al., 2017; Schumann et al., 2014; Heringer et al., 2014; Tilkat et al., 2014; Polzin et al., 2014; Ramos-Castellá et al., 2014; Ramírez-Mosqueda et al., 2016; Wilken et al., 2014). Also, this technique was found to generate low rates of somaclonal variation compared to culture in solid or semi solid media (Shenoy and Vasil, 1992); although it can induce some physiological disorders like vitrification.

Somaclonal variation can be heritable through genetic pathway or non-heritable following epigenetic modifications (Larkin and Scowcroft, 1981). To detect genetic variations, several DNA markers can be used. Among PCR-based techniques, Inter Specific Sequence Repeat (ISSR) is one of the simplest and most common. Indeed, it involves the amplification of DNA segments present at an amplifiable distance between two identical microsatellite repeat regions oriented in the opposite direction. Such markers were successfully applied in many species such as *Phoenix dactylifera* L. (Kumar et al., 2010), *Oryza sativa* L. (Frederic et al., 2007) and *Prunus mume* cultivars (Guo et al., 2007).

The aims of the present study were: i) to establish a reliable protocol for the *in vitro* propagation of Myrobolan using RITA® bioreactors and low PGR concentrations; and ii) to evaluate the genetic conformity of micropropagated plantlets by ISSR genetic markers.

2. Material and methods

2.1. Plant material

Juvenile one-node cuttings were collected from Myrobolan plants growing in the South of Tunisia (Regueb-Sfax) in spring time (March-July).

2.2. Explant disinfection

Solutions of sodium hypochlorite (12%) supplemented with Tween 20 and mercuric chloride ($HgCl_2$) were used at various concentrations. Then, explants (1–1.3 cm of length) were washed three times with sterile distilled water and aseptically transferred into the initiation medium.

2.3. Culture media composition and culture conditions

Our Initiation Medium was made of a half-strength MS medium (½MS) (Murashige and Skoog, 1962) supplemented with 30 g L^{-1} sucrose and 2 g L^{-1} Gelrite. The pH of the culture medium was adjusted to 6.2 before autoclaving (120 °C/1 bar/20 min). Culture tubes containing 20 mL of medium were placed in a culture room where temperature was kept at 25 °C. Vessels were illuminated with fluorescent tubes at a light intensity of 30 µmol m^{-2} s^{-1} under a 14 h/24 h photoperiod. Cultures were maintained under these conditions for 12 weeks at each stage.

2.4. Shoot multiplication using rita® bioreactors

At first step, young vitroplants (2–3 cm) were cultured in tubes containing ½MS and each culture tube contained one vitroplant. Different concentrations of BAP were supplemented (1, 2, 3 and 4 mg L^{-1}) in order to determine the most adequate concentration for multiplication.

RITA® bioreactors based on temporary immersion were used for the proliferation of Myrobolan vitrocultures. Indeed, vitroplants were grown in RITA® bioreactors using 200 mL of MS medium supplemented with 30 g L^{-1} of sucrose. The pH was adjusted to 5.8 before autoclaving (120 °C/1 bar/ 20 min). The RITA® system is composed of two parts separated by a filter. Vitroplants were placed onto the filter and the liquid medium in the basal compartment.

Several parameters of temporary immersion of Myrobolan were tested: Immersion frequencies (every 3, 6 and 12 h) and time of immersion ranging from 5 to 60 min per 12 h were tested in order to select the best duration of immersion and different concentrations of auxin (IBA) and cytokinin (BAP) were tested to optimize the *in vitro* development of Myrobolan vitroplants in RITA® bioreactors. In this case, excised plantlets were transferred onto liquid MS supplemented with 1, 2, 3 and 4 mg L^{-1} IBA (indole-3-butyric acid) to choose the best concentration for the initiation of root formation and elongation of shootlets. All cultures were incubated at 25 ± 2 °C under a 14 h/24 h light/dark photoperiod with a light intensity of 30 µmol m^{-2} s^{-1}. Eight vitroplants per container were used per immersion system (with three replicates each). After 6 weeks of culture, the length of shoots, number of leaves, number of buds, fresh weight and the number and length of roots per container were determined. Also, the content of photosynthetic pigments (chlorophyll *a*, b, a + b, and carotenes) was evaluated (Arnon, 1949; Porra et al., 1989).

Several parameters such as hyperhydration, growth, browning and necrosis were sought. Each system was kept in culture room during 8 weeks.

2.5. Shoot elongation and rooting

In order to optimize the *in vitro* rooting of shootlets, vitroplants were taken out of RITA® bioreactors and cultivated in 150 mL glass jars with 15 mL of liquid MS medium supplemented with 30 g L^{-1} sucrose and 1 g L^{-1} activated charcoal, in a solid MS medium comprising 1 g L^{-1} activated charcoal and in RITA® bioreactors also containing only 1 g L^{-1} activated charcoal diluted in 200 mL MS medium, without any auxin, under the same conditions optimised in the multiplication stage. At this stage, physiological parameters of Myrobolan vitroplants were tested under light/dark conditions and in a culture environment containing 1 g L^{-1} activated charcoal. All cultures were incubated at 25 ± 2 °C under a 14 h/24 h light/dark and a light intensity of 30 µmol m^{-2} s^{-1} using white-light lamps. Shoots were incubated in dark conditions using an incubator during 40 days. Eight vitroplants per container were used per immersion system (with three replicates each). After 6 weeks of culture, the length of shoots, the number of leaves and the number and length of roots were determined. Additionally, the content of photosynthetic pigments (chlorophyll a, b, a + b, and carotenes) was evaluated (Arnon,1949; Porra et al., 1989).

The *in vitro* hardening stage of vitroplants was obtained using an MS medium deprived of PGRs for 12 weeks of incubation.

2.6. Vitroplant acclimatization

Rooted vitroplants of 8–11 cm height and showing at least two developed roots were subsequently immersed in 1% fungicide solution (Palt 44), then washed thoroughly with tap water. Microplants were then planted in 500 mL disposable pots containing a mixture of horticultural substrate and pure sand (2:1 v/v), then placed in a greenhouse. During the first two months of acclimatization, plants were kept under