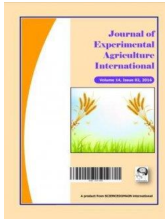

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Germination and Development of 'Precocinho' Peach Embryos: Asepsis and Use of PPM^{TM} in Culture Medium

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Authors' contributions

This work was carried out in collaboration between all authors. Authors DCN and MCBR designed the study, wrote the protocol and the first draft of the manuscript. Authors DCN, MD and SC performed the statistical analysis and managed the analyses of the study. Author DCN managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Embryo culture is a technique used to rescue immature embryos which would not germinate under natural conditions. However, one of the biggest problems of this process is the contamination by microorganisms. The objective of this work was to test asepsis methods and the use of PPM^{TM} (Plant Preservative Mixture^{TM}) in the culture medium for the germination and development of 'Precocinho' peach embryos. After extraction from the fruits, seeds were submitted to treatments with thimerosal, sodium hypochlorite (NaClO), flame sterilization and moxifloxacin hydrochloride, in culture medium with and without addition of PPM^{TM}, totaling nine treatments. The experimental design was completely randomized, with 20 replicates per treatment. Three evaluations were performed: *in vitro* establishment (at 55 days of cultivation), embryo development (at 65 days of cultivation) and acclimatization (20 days after transplanting in the greenhouse). Six treatments did

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not had any contamination, on the first evaluation. However, 65 days after culture, the NaClO with PPM™ addition presented the best results for the variables analyzed. The average seedling survival was 81.6%, after acclimatization. The use of sodium hypochlorite with the addition of PPM™ is shown as the most efficient treatment, among the tested ones, for the germination and development of 'Precocinho' peach embryos.

Keywords: Prunus persica (L.) Batsch; embryo culture; immature embryos; disinfestation.

1. INTRODUCTION

Peach breeding programs have been carried out hybridizations to obtain very early ripening cultivars, in order to satisfy growers' and consumers' demands. Brazilian breeding programs are responsible for about 90% of the planted cultivars in the country, being the most important programs those from the Agronomic Institute of Campinas (SP) and Embrapa Temperate Climate, in Pelotas (RS) [1].

Seeds from mother plants whose fruits have a short fruit development period may present germination problems because the embryo is immature at the time of harvest. However, embryo culture is a technique that makes possible the germination and development of immature embryos, being essential in a breeding program [2,3,4]. Without this technique, early ripening peach cultivars could not be used as parents, since the percentage of germination would be very low [5]. The fruit development period of 'Precocinho' is approximately 100 days, being considered an early cultivar in the growing conditions of southern Brazil [6], presenting the characteristic of immature embryos. In addition, plants from the embryo culture process are more vigorous than those from the conventional methods, since the embryo rescue is made in the lab, under controlled conditions of temperature (23-25ºC), photoperiod (12-16 h) and culture medium suitable for the development of embryos [7].

In tissue culture in general, contamination by microorganisms is one of the major problems which occurs because factors, such as culture medium and temperature, create a favorable environment for bacteria, yeasts and fungi growth. The contamination is established in the culture medium or in the explant, and competes with the plant for nutrients. Also, the microorganisms may release toxic substances in the medium, which may inhibit the development of the explants leading to loss of material [8,9].

Several substances with germicidal action are used to disinfect plant material [9]. Among the most used products for explants asepsis is the sodium hypochlorite (NaClO), normally used in a concentration of 2%, from 5 to 30 minutes of immersion. The NaClO has the advantage of being water soluble, which makes it easy to remove and avoid the residual effects [10]. The process of cleaning the explants surface with disinfectant solutions may not be sufficient to completely remove the microorganisms, especially in the case of endogenous bacteria. The usage of antibiotics in the medium is possible, however, its efficiency is still questionable, because in addition to the possibility of inducing resistance of some bacteria [11], these may reappear after transferring the explants to the culture medium without antibiotics [12].

The PPM™ (Plant Preservative Mixture™) it is a mixture of broad spectrum antibiotics that have been used in plant tissue culture since 1996, being effective in reducing *in vitro* contamination for some species [13]. It has the advantage over other antibiotics because it is heat stable which means that it can be added to the medium and autoclaved [14].

The degree of disinfestation, the combinations of the active principles and the response of the explants can be highly variable [15,16]. Thus, a study to adequately address the disinfestation protocols, according to the species, and elucidates the sensibility of the tissue to the process, it is needed [17].

The aim of this work was to test asepsis methods and the use of PPM™ in the culture medium for germination and development of 'Precocinho' peach embryos.

2. MATERIALS AND METHODS

The experiment was carried out at Embrapa Temperate Agriculture, Pelotas (RS), Brazil, between November 2016 and February 2017.

Fruits from Precocinho peach cultivar obtained by open pollination in 2016/2017 harvest season, were taken to the laboratory and treated with 70º alcohol for five minutes. Seeds from 180 fruits were extracted by cutting them (including the endocarp) on the suture line and removed with a sterilized forceps. Once extracted from the fruits, the seeds were submitted to the following asepsis treatments: T1 = thimerosal (1500 ppm, immersion for 5 minutes), T2 = NaClO (0,5%, immersion for 5 minutes), T3 = flame sterilization (immersion in alcohol 96°, and flame sterilization for 3 seconds), T4 = moxifloxacin hydrochloride (0,2%, immersion for 5 minutes); T5 = thimerosal (1500 ppm, immersion for 5 minutes T6 = NaClO (0,5%, immersion for 5 minutes), T7 = flame sterilization (immersion in alcohol 96°, and flame sterilization for 3 seconds), T8 = moxifloxacin hydrochloride (0,2%, immersion for 5 minutes; T9 = thimerosal (1500 ppm, immersion for 5 minutes), kept for five days under 22±1°C temperature). Treatments T1 to T4 and T9 seeds were planted in culture medium with addition of 1ml L^{-1} of PPM^{TM} whereas T5 to T8 seeds were planted in culture medium without PPM^{TM}.

After asepsis, the seeds were replaced in the test tubes with WPM culture medium (Wood Plant Medium) [18], supplemented with 30 g L^{-1} of sucrose and 7 g L^{-1} of agar. The material (except T9) was placed in a cold room at 4±1ºC temperature.

The experimental design was completely randomized with 20 replications and each test tube with a seed, was considered as an experimental unit.

After 55 days of vernalization, when the material was removed from the cold room, the percentage of contamination and survival, presence of aerial part and root length were evaluated.

After this first evaluation the test tubes were transferred to a growth chamber with 24±1°C temperature and 16h photoperiod, where they remained for ten days.

At 65 days after incubation, the embryo development was evaluated. The seedlings were removed from the test tubes. Plantlets' aerial part and main root length were measured and the number of secondary roots was counted.

Subsequently, seedlings of normal development, with proportional growth between aerial part and root system, were planted in plastic trays, with substrate (peat, sand and vermiculite 1:1:1) and left to grow in a greenhouse. After 20 days the percentage of plant survival and plant height were evaluated.

Data were analyzed by analysis of variance (ANOVA), and means were subjected to the Scott-Knott grouping test (*P* = .05) [19].

3. RESULTS AND DISCUSSION

Soon after vernalization, all tested treatments showed high percentages of survival, between 90 and 100% (Table 1). The period of fruits storage greatly influences the survival and formation of seedlings in the embryo culture, and they emphasize the importance of performing the embryo rescue soon after harvesting [20]. In the present work the experiment was performed in the same week of the fruit harvesting in the field.

Table 1. Main root length in cm, percentage of survival, contamination, germination and aerial part presence in peach seedlings 'Precocinho' after embryo culture and vernalization

Treatment	Main root length (cm)	Survival (%)	Contamination (%)	Germination (%)	Presence of aerial part (%)
T6	0.79 a[1]	100	0	95	60
T9	0.78 a	95	5	90	65
T4	0.58 b	100	5	85	75
T2	0.50 b	90	0	75	100
T5	0.48 b	95	0	55	35
T8	0.44 b	90	40	45	20
T7	0.42 b	95	0	65	40
T3	0.37 b	100	0	65	75
T1	0.32 b	100	0	60	30

[1]Means followed by the same letter, in the column, belong to the same group by the Scott-Knott grouping test (P = .05) T1 = thimerosal, T2 = NaClO, T3 = flame sterilization, T4 = moxifloxacin hydrochloride, T1 to T4 with PPM^{TM}; T5 = thimerosal, T6 = NaClO, T7 = flame sterilization, T8 = moxifloxacin hydrochloride, T5 to T8 without PPM^{TM}; T9 = thimerosal, with PPM^{TM}, kept five days under 22±1°C

For percentage of contamination, the T8 (moxifloxacin hydrochloride, without addition of PPM™) was the least efficient, with 40% of the tubes with contamination. The seeds appeared normal (shape and color), being counted as live seeds, however, they were the ones with the lowest percentage of germination (45%). The treatments T1, T2, T3, T5, T6 and T7 did not present contamination, being effective in this initial stage of establishment *in vitro*. Using the disinfestation process with alcohol 70% (1 min.) and NaClO (1.25%, 15 min.), [21] also had 100% survival and zero contamination, in the *in vitro* establishment of shoot apex of GF-677 peach rootstock. In relation to the use of PPM™, [22] found lower percentages of bacterial contamination (8.3%) using higher doses of PPM™ (4.0 ml L^{-1}) in the asepsis of *Pyrus communis* explants for *in vitro* establishment. In this study the asepsis procedure significantly influenced the percentage of seed contamination, while the PPM™ in the culture medium did not have any significant effect. The treatments T5, T6 e T7 showed these results, since they were those that did not have PPM™ addition to the medium and, nevertheless, they did not present contamination (Table 1).

The treatments T2, T4, T6 and T9 had satisfactory germination percentage, between 75 and 95%. However, stand out the T2 (NaClO, with PPM™), which, at the initial evaluation, all the explants already presented an early development of the aerial part. Analyzing culture media in early nectarine embryos, [23] reported 100% germination using WPM medium in vermiculite substrate to *in vitro* seed germination. However, [24] testing peach embryo culture and direct seed stratification had an average germination percentage of 50.08%, higher in embryo culture (4.49). The T1 (thimerosal, with PPM™) and T5 (thimerosal, with no PPM™) did not present contamination, however, are among the treatments with lower percentage of germination and presence of aerial part.

As for the main root length (pivotant root) there were significant differences between treatments (P = .0001). The treatments T6 (NaClO, without PPM™) and T9 (thimerosal, with PPM™, kept for five days under 22±1°C) presented better results (0.79 and 0.78cm, respectively), and did not statistically differ from each other (Table 1). The other treatments had inferior results concerning to this variable, and had no statistical differences among them, with root length between 0.32 and 0.58cm.

In the second evaluation, after incubation, when the development of the embryos were analyzed, the treatment T2 (NaClO, with PPM™) presented the best results for the analyzed variables, not statistically differing from T3, T4 and T6 for main root length and from T3 and T6 for the number of secondary roots (Table 2). In general, the best embryo development was obtained in the treatments with sodium hypocloride (T2 and T6). The sodium hypochlorite was also efficient in disinfestation and *in vitro* germination of other species such as guabiju [25] and jabuticaba [26]. Sodium hypochlorite, because of its oxidant potential, can increase the supply of oxygen for the seed, this would have influenced the increase of germination percentage in canjarana seeds, when sodium hypochlorite in different concentrations was tested [27].

One of the most important conditions for development of *in vitro* plants, is the formation of a well-developed, uniform, root system, which provides good seedlings support, allowing their survival during the acclimatization stage [28]. In the present work, the treatments T2, T3 and T6 presented uniformity in the length of the main root, between 7.20 and 7.56cm and in number of secondary roots, between 11.15 and 12.45, considered as satisfactory (Table 2).

For the length of aerial part, the lowest indexes obtained were in plantlets submitted to T8 (moxifloxacin hydrochloride, without PPM™) and T9 (thimerosal, with PPM™, kept for five days under 22±1°C) treatments. All the other treatments had shoot height between 2.93 and 3.98cm, and did not statistically differ among them.

The T8(moxifloxacin hydrochloride, without PPM™) was the treatment that presented the lowest indexes in all observed parameters. This is probably due to the fact that this treatment presented the highest percentage of contamination in the initial stage of the cultivation, which may have jeopardized the development of the embryos.

Regarding acclimatization, an overall mean seedlings survival of 81.6% was obtained. Seedlings from T6 (NaClO, without PPM™) and T5 (thimerosal, without PPM™) presented 95% survival (Table 3). In another work with acclimatization of micropropagated seedlings of the rootstock Mirabolano 29C (*Prunus cerasifera*) [29], obtained survival averages between 19.4

Table 2. Root length, shoot height and number of secondary roots of 'Precocinho' peach seedlings after incubation

Treatment	Root length (cm)	Shoot height (cm)	Nº secondary roots
T2	7.56 a[1]	3.98 a	11.60 a
T6	7.30 a	3.38 a	11.15 a
T3	7.20 a	3.45 a	12.45 a
T4	6.65 a	3.25 a	8.15 b
T1	5.76 b	2.90 a	8.05 b
T7	5.55 b	2.79 a	7.05 b
T5	5.54 b	2.93 a	7.95 b
T9	4.62 b	1.77 b	4.89 b
T8	4.03 b	2.22 b	5.15 b
P-value	0.0015	<0.0001	0.0003

[1]Means followed by the same letter, in the column, belong to the same group by the Scott-Knott grouping test (P = .05) T1 = thimerosal, T2 = NaClO, T3 = flame sterilization, T4 = moxifloxacin hydrochloride, T1 to T4 with PPM™; T5 = thimerosal, T6 = NaClO, T7 = flame sterilization, T8 = moxifloxacin hydrochloride, T5 to T8 without PPM™; T9 = thimerosal, with PPM™, kept five days under 22±1°C

and 44.4%. The good result in the acclimatization stage, obtained in the present study, is probably related to the good quality of the aerial part and the root system formed during in vitro development [30].

Table 3. Shoot height and percentage of survival in acclimatization of 'Precocinho' peach seedlings

Treatment	Shoot heigh (cm)	Survival (%)
T7	8.38 a[1]	85.0
T6	7.88 a	95.0
T2	7.48 a	85.0
T3	6.85 a	64.5
T4	5.53 b	82.5
T5	5.48 b	95.0
T1	5.23 b	90.0
T8	5.05 b	75.0
T9	2.68 b	62.0

[1]Means followed by the same letter, in the column, belong to the same group by the Scott-Knott grouping test (P = .05) T1 = thimerosal, T2 = NaClO, T3 = flame sterilization, T4 = moxifloxacin hydrochloride, T1 to T4 with PPM™; T5 = thimerosal, T6 = NaClO, T7 = flame sterilization, T8 = moxifloxacin hydrochloride, T5 to T8 without PPM™; T9 = thimerosal, with PPM™, kept five days under 22±1°C

As for the main shoot height there were significant differences between treatments (*P* = .0039). The seedlings of the treatments T2, T3, T6 and T7 presented the best results, between 6.85 and 8.38cm, not statistically differing among them. Acclimatization is considered the most delicate step in the process of obtaining seedlings by tissue culture. During the *in vitro* cultivation, the seedlings are conditioned in an enclosed environment under controlled temperature (24 ± 1°C) and photoperiod (16 h), high air humidity in addition to the supply of nutrients. Therefore, when removed from these conditions and transferred to *ex vitro* environment, this material is prone to dehydration [31,32].

The asepsis treatments, which the seeds were submitted prior to *in vitro* establishment, probably did not influence the seedling development after acclimatization. However, it is important to take into account the treatments with lower contamination, higher percentage of survival and germination in the initial stage and better embryo development in order to obtain a higher number of good seedlings so that they can be acclimatized later.

4. CONCLUSION

The use of sodium hypochlorite and flame sterilization were the most efficient asepsis treatments for the germination and development of peach embryos, among the tested ones.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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