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Conservation

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Safeguard of emilia apple germplasm (*malus communis l. Subsp.* Yellow pippin of blenheim) by in vitro tissue *grolwing*

Salvaguarda del germoplasma para manzana emilia (*malus communis l. Subsp.* Reineta amarilla de blenheim) por cultivo de tejidos *in vitro*

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Abstract

It is of great importance to maintain the genetic diversity of traditional materials and wild plants. The Emilia apple germplasm was safeguarded, using in vitro tissue culture techniques. Two culture media and six concentrations were evaluated for disinfection; in addition, eight treatments for budding, using different concentrations of 6-benzylaminopurine (BAP) and for rooting four treatments with indole butyric acid (IBA). We used a DCA with a 2 x 4 factorial arrangement with six observations. The percentages of contamination, oxidation, viability, days at sprouting, number of shoots and length of shoots were evaluated. Treatments equal to and greater than 10% NaClO for 10 minutes had adequate values of non-contamination of explants. The lower number of oxidized explants had a direct proportional correlation in the responses to less concentration and time of exposure. The highest percentage of viable explants (77%) was expressed in 10% NaClO treatment for 15 minutes. The best responses regarding the variable days to budding were presented in the treatments without BAP intervention. The best response in the number of outbreaks was manifested for the 1 ppm BAP treatment in the culture media (Murashige and Skoog modified - MSM and Modified CHU 10 by Gerloff). The best response in the size of the outbreak was expressed in the treatment CHU + 1 ppm BAP. The cellular tissues the Emilia apple show high susceptibility to microbial contaminants and the oxidation of tissues, which makes it difficult to multiply by in vitro culture. High concentrations of BAP inhibit spontaneous physiological responses in explants of the Emilia apple, especially in the early stages where there is greater production of intrinsic hormonal effects.

Keywords: Culture media, plant hormones, botanical conservation, micropropagation.

Resumen

Es de gran importancia mantener la diversidad genética de los materiales tradicionales y de plantas silvestres. Se realizó la salvaguarda del germoplasma de manzana Emilia, utilizando técnicas de cultivo de tejidos in vitro. Se evaluaron dos medios de cultivo y seis concentraciones para desinfección; además, de ocho tratamientos para brotación, utilizando distintas concentraciones de 6-bencilaminopurina (BAP) y para enraizamiento cuatro tratamientos con ácido indol butírico (IBA). Se utilizó un DCA con un arreglo factorial 2 x 4 con seis observaciones. Se evaluó: porcentajes de contaminación, de oxidación, viabilidad, días a la brotación, numero de brotes y longitud de brotes. Los tratamientos iguales y superiores al 10% de NaClO durante 10 minutos presentaron valores adecuados de no contaminación de explantes. La menor cantidad de explantes oxidados presentaron correlación directamente proporcional en las respuestas a menor concentración y tiempo de exposición. El mayor porcentaje de explantes viables (77%) se expresó en el tratamiento de NaClO al 10% durante 15 minutos. Las mejores respuestas en cuanto a la variable días a la brotación se presentó en los tratamientos sin intervención del BAP. La mejor respuesta en cuanto al número de brotes se manifestó para el tratamiento 1 ppm de BAP en los medios de cultivo (Murashige y Skoog modificado - MSM y Medio CHU 10 Modificado por Gerloff). La mejor respuesta en el tamaño del brote se expresó en el tratamiento CHU + 1 ppm de BAP. Los tejidos celulares de la manzana Emilia presentan alta susceptibilidad a contaminantes microbianos y a la oxidación de tejidos, por lo cual dificulta la multiplicación por medio de cultivo in vitro. Las altas concentraciones de BAP inhiben respuestas fisiológicas espontáneas en explantes de la manzana Emilia, especialmente en etapas iniciales donde existe mayor producción de efectos hormonales intrínsecos.

Palabras claves: Medios de cultivo, fitohormonas, conservación botánica, micropropagación.

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1 Introduction

Plant genetic resources are the basis of food sovereignty; it is therefore of the utmost importance to maintain the genetic diversity of traditional and regional varieties, improved cultivars and wild plants. Genetic diversity provides farmers and breeders with options to develop new cultivars or hybrids, which may be more productive, have better characteristics of fruit, flower or plant structure, levels of resistance or tolerance to pathogens and/or unfavorable environmental conditions.

Although there are more than 10,000 documented apple cultivars worldwide and the apple production area is widely spread geographically, world production is dominated by relatively few cultivars, many of which are closely related (Janick and Moore, 1996; Hokanson et al., 2001). In the last century, despite the existence of a large number of worldwide apple breeding programs, only a few genotypes have been well adapted (eg Red Delicious", "Golden Delicious", "Jonathan", McIntosh and Cox's Orange Pippin, which have been widely used in breeding programs to release new varieties with desirable traits (Noiton and Alspach 1996, Hokanson et al 2001, Laurens et al. 2010). The gradual replacement of traditional and locally well-adapted cultivars by a few widespread modern varieties has led to a dramatic loss of genetic diversity in orchards and may hinder future plant reproduction (Laurens et al., 2010).

The genetic diversity of crops is being lost at an alarming rate; this loss raises socioeconomic, ethical and political issues (Esquinas, 2005). At the beginning of the 20th century in Spain, as in the rest of Europe, cultivated apple trees were preferably traditional local varieties, which together with wild forms represented the genetic diversity of the crop (Itoiz, 2000). In the 1960s new American varieties of apple were introduced into America, Spain and the rest of Europe, from family gardens, where several species and many varieties were grown, to monovarietal plantations or at most one as main and another as pollinator. In recent years, numerous indigenous varieties have disappeared, although they suffer from effects such as low yields, low fruit sizes and irregular harvests, they have values of great importance such as adaptation to the environment, levels of resistance to diseases and organoleptic quality. This process of loss of variability, of genes, by the pressure of the new cultivars is what is called genetic erosion. In addition, agricultural industrialization, green revolution technologies, environmental changes and civil conflicts, have been cited as contributors to the erosion of the biodiversity of native apple crops (Goland and Bauer, 2004).

The varietal changes has been so rapid that many varieties, native or not, have disappeared without a trace of their genes and can never be recovered, unless they are obtained by means of genetic engineering or by resorting to germplasm banks. Genetic erosion is a worldwide continuous and widespread process (Priolli et al., 2004).

Changes in the genome may involve changes in the number of chromosomes (genomic mutations), in some chromosomes (chromosomal mutations), or in some gene (gene mutations). It is known the prolific agogic genealogy of the Delicious variety whose original clone was discovered at the end of the 19th century and today more than 200 mutations are known from the original or from some of its mutants (Forsline et al., 2003).

The apple variety Emilia is an emblematic and representative fruit of the province of Tungurahua-Ecuador. It has come to be conceived as a symbol of culture and tradition among its peoples. In decades the cultivation has been reduced by the import of fruit of other varieties, low productivity, neglect by authorities and volcanic continue eruptions; factors that lead to the loss of fruit diversity in the country (Lara, 2009).

Red and green unicorn varieties today account for more than half of per capita consumption of apple. These varieties have almost completely displaced the traditional ones with the corresponding risk of their disappearance that, in addition is increased by the fact of the increasing abandonment or the urbanization of many farms. To avoid this problem, or at least minimize the thiis loss, the creation of germplasm banks is being encouraged for the conservation of genetic material, which may be of great use in the near future (Laurens et al., 2010).

The maintenance of the material and the genetic variability of the collections can be done in situ or ex situ. In situ conservation means that it is carried out in natural areas where the germplasm has naturally developed, and in the case of cultivated varieties, in the vicinity of the area where they have acquired their distinctive properties. On the other hand, this type of banks also has the disadvantage that a lot of space and maintenance work is necessary and, therefore, there have been proposed new alternati-

ve methods of conservation, such as cryo conservation of resting buds or conservation of plants in vitro (Towill et al., 2004). The National Plant Germplasm System has carried out viability trials of apple seed preserved in liquid N2 since 1992 and after eight years of storage it has not observed a viability decrease as they have managed to recover 98% of the stored varieties of this form (Forsline et al., 2003).

Micro-propagation involves the large-scale multiplication of a genotype through the use of in vitro culture techniques. In vitro cultivation is a tool for breeding, in which plants of uniform quality are generated on a commercial scale, from a select genotype and with an unlimited multiplication rate (Druart, 1997). The apple micro-propagation has played an important role in: production of healthy plants, disease-free and in the rapid multiplication of cuttings and porta-grafts with desirable characteristics; success obtained through pre-existing meristems (cultivation of apical buds or nodal segments), being the technique that has successfully transcended the experimental areas into practice (Engelmann, 2011).

Tissue culture involves the growth of cells and tissues in vitro under aseptic conditions. It is a very useful tool since, once the crops are established, they are available at any time for the researcher and the time needed to carry out specific experiments can be reduced considerably when compared to the use of whole plants. The relative homogeneity of the crops and the ease with which the conditions can be standardized (composition of the culture medium, nutritional parameters, levels of growth regulators, addition of effectors), which promote the reproducibility of results (Baajji et al. Druart, 2011).

To provide the explants with adequate replication conditions in vitro, techniques have been developed to maintain high diversity in confined spaces, under aseptic conditions and safe from environmental hazards that could lead to loss (Hao et al., 2001) . It is possible the multiplication thanks to the property of totipoteny that the vegetal cells have, this is the capacity to regenerate a complete plant when it is subject to suitable stimuli, thus the somatic cells of any fabric could form stems, roots or somatic embryos according to the competition they have and the stimulus they receive (Engelmann, 2011).

Among the main problems that could present the techniques of in vitro culture, is the Phenolization; In the same the explants frequently are brown or blackish soon after the isolation. When this occurs it inhibits growth and the tissue generally dies. Young tissues are less susceptible to darkening than the more mature ones (Bajji and Druart, 2011). Vitrification, also called hyperhydration or translucency, occurs in plant tissues that have a large amount of water, young and tender plant material is more susceptible to vitrification and the degree of translucency is associated with high levels of cytokinins, deficiency in gas exchange, and low luminic irrigation, high temperetures, and low concentration of agar and sugars of the medium (Gagliardi et al., 2003). The incidence of in vitro pathogens is one of the most important problems in the development of plant biotechnology, especially in cell and tissue culture. The effect of contaminating microorganisms on vitro plants can be considerable taking into account that they compete for the nutrients of the environment and cause direct and indirect damages by the colonization of their tissues and by the expulsion to the environment of toxic metabolites, causing a reduction to the multiplication coefficients, inhibiting rooting and causing the explant to die (Habiba et al., 2002).

The culture media must be provided with physical and chemical elements that constitute the nutritive substance of diverse consistency (solid to liquid), which provide nutrition and stimulation in the development of the explant. The auxins comprise a large family of substances that have in common the ability to produce an enlargement and cellular elongation. The most used in tissue culture are indolacetic acid (AIA), naphthaleneacetic acid (ANA), 2,4-D, picloram (FernÃ¡ndez et al., 2009). For the use of the Auxins it is not possible to establish a particular concentration that must be handled in a single case. However, AIA is generally used in concentrations ranging from 0.001 to 10 ppm, with an optimum point about 0.1 to 1 ppm; 2,4-D is used in concentrations ranging from 0.1 to 10 ppm, with an optimum point which is frequently found around 1 to 5 ppm. The ANA is generally used in concentrations slightly higher than 1 to 10 ppm, with an optimum of about 2 ppm (Martin, 2003).

Cytokinins are often used to stimulate growth and development; the most common being Kinetin, N6-benzyladenine (BA), N6-isopentenyl adenine (2iP), and 6-benzylaminopurine (BAP). They usually stimulate cell division. They promote the formation of axillary stems, because they diminish

the apical dominance; also slow down aging. They are used in research between 0.01 and 10 ppm. In many culture media, Adenine Sulfate is beneficial and is often added at a rate of 40 to 120 ppm. All these materials should be prepared in advance and kept refrigerated as concentrated solutions (Gao et al., 2009).

In search of new alternatives that contribute to the agricultural economy of the central zone of Ecuador and the consequent safeguard of one of the main representative cultivated fruits, as is the apple, Emilia variety (Malus communis subsp. Reineta Amarilla de Blenheim), which presents desired qualities by the final consumer such as: juiciness, sweetness, taste and local identity, this research was carried out.

1.1 Materials and Method

The used explants were collected from mixed fruit orchards located in the Huaynacuri neighborhood, San Miguelito parish, Town PÃllaro, Tungurahua province, Ecuador (1 ° 13 '63' 'Lat. And 78 ° 32' 8 " Long.). Samples of buds selected in situ from supplier or explant donor plants were collected, which showed desired phenotypic characteristics such as: numerous shoots of apical buds, perennial and dense foliage, and vigorous plants, visibly healthy, free of bacteria, fungi, viruses and mites. The specimens were collected and placed in Ziploc®plastic bags with a small amount of sterile distilled water to avoid wilting and reduce the physiological deterioration of the plant material. The bags were protected with carboard packs to avoid mechanical damage during transport to the laboratory.

The work was carried out in the Laboratory of Agricultural Biotechnology of the Faculty of Agricultural Sciences of the Central University of Ecuador (FCA-UCE), University Campus in Quito, the sowings were performed in laminar flow chamber, the tests remained in the Growing room under controlled growth conditions. The variables evaluated in the explants were: percentage of contamination, percentage of oxidation, percentage of viability, days at sprouting, number of shoots (at 60 days after peal), length of spout (60 days of peal, measured from the base of the bud to the apical bud), and percentage of rooted shoots.

In the laboratory, apical buds were selected in the best condition, the fresh stem and damaged parts were removed, segments 40-50 mm long were cut, these buds were placed in beakers with distilled water until the time they started the disinfection phase. Once the explants were obtained, two washes were carried out with drinking water and 2 mL 100 mL^{-1} of Tween®20. It was kept under constant stirring for 10 minutes, then three rinses were performed using distilled water. Then, the explants were immersed in independent solutions of: Mancozeb (2.5 g 500 mL^{-1}) and Copper Sulfate Pentahydrate (3 mL 500 mL^{-1}) in water, respectively; In both cases for 20 minutes under stirring in a heating plate, after which three washings of two minutes each were performed using distilled water. In the Labconco®Vertical Laminar Flow Hood 3970424 the explants were introduced into 70% ethanol for 30 seconds, then three minute rinses were performed using sterile distilled water, then the explants were immersed in Sodium Hypochlorite, testing different disinfection treatments. After this, three rinses were carried out with sterile distilled water of one minute each, to finally immersion the explants in a solution of Citric Acid (0.3 g L^{-1}) during the time that the planting lasts.

The modification to the MSM medium was the addition of Vitamin B5 and the Medium CHU 10 was maintained as was modified by Gerloff. The variables evaluated for the explants were: Percentage of Contamination (the presence of fungi and bacteria in the culture medium or explant was determined), Oxidation, Feasibility; all valued, for 3 weeks - once a week. Three replicates were established at different times for each variable.

Bioassays were used for the evaluation of contamination in explants using 3 g L^{-1} of commercial detergent (components: surfactants, enhancers, enzymes, bleaches, perfumes, fillers and optical brighteners) plus the addition of 6 mL 100 mL^{-1} of Tween 20®and at the end of the washing process 70% ethanol was applied for 30 seconds. Different concentrations of Sodium Hypochlorite were tested in incremental times. As for the budding phases, two types of culture medium with different concentrations of BAP hormone (Table 1) were evaluated.

For the statistical analysis of the data the Chi square test was used for the first phase (bioassay adaptation). Fifteen observations were made; for the second phase (bio-rooting trials), a Completely Random Design (CRD) was used with a 2 x 4 factorial arrangement with six observations and descriptive statistics for the third phase (substrate adapta-

Table 1. Distribución de las tierras de estudio en términos de su provincia y si pertenecen o no al Socio Bosque

Explant codification	Description	Shoot codification	Description
t1	5 % NaClO + 10 min.	**t1**	CHU + 0 ppm de BAP
t2	5 % NaClO + 15 min.	**t2**	MSM + 0 ppm de BAP
t3	10 % NaClO + 10 min.	**t3**	CHU + 0,5 ppm de BAP
t4	10 % NaClO + 15 min.	**t4**	MSM + 0,5 ppm de BAP
t5	15 % NaClO + 10 min.	**t5**	CHU + 1 ppm de BAP
t6	15 % NaClO + 15 min.	**t6**	MSM + 1 ppm de BAP
		t7	CHU + 1,5 ppm de BAP
		t8	MSM + 1,5 ppm de BAP

tion), with six observations. Statistical analysis was performed using the statistical software SPSS version 2.0. Tukey's HSD test with 5 % significance was used to determine the mean difference.

2 Results and discussion

2.1 Percentage of contamination

The lowest percentage of contamination in explants (17 %) was obtained with t6 treatment (15 % NaClO for 15 minutes); Unlike the t1 treatment (5 % NaClO for 10 minutes) which generated 63 % contamination was the highest response. Values equal to or greater than 10 % NaClO for a period of 10 minutes give constant responses regarding the presence of uncontaminated explants (Graph 1).

Bajji and Druart (2011) determined that with sodium and calcium hypochlorite, disinfection of Prunus explants derived from adult trees grown under field conditions is not effective compared to mother plants growing under greenhouse conditions. Another factor to take into account is the pubescence of the tissue. Habiba et al. (2002) stated that ïf the tissue is pubescent, a pre-wash with detergent or 70 % ethanol should be done for 30 seconds to break the surface tension and make it accessible to the action of the disinfectant agents..As a part of bioassays good results were obtained by performing two prewashes with the commercial detergent and the addition of Tween 20®.

Druart (1997) mentions that the used disinfection methods do not always eliminate bacterial populations associated with plant tissues in vivo. Many are able to remain latent in the intercellular spaces or in the conductive beams, being protected of the chemical agents. Also the contamination could be related to the presence of endogenous fungi acquired by the plant in its natural environment.

2.2 Percentage of oxidation

The oxidation variable was analyzed in each of the treatments, where it was shown that the treatment t1 (5 % NaClO x 5 minutes) reached the highest percentage of free oxidation explants (80 %), followed by t4 treatment (10 % NaClO x 15 Minutes) (Figure 2). Chlorine acts as a potent bactericide because hypochlorous acid forms when mixed with water and the oxygen released in this reaction is a very strong oxidizing agent, at the same time that the microorganisms are destroyed as it can modify functional groups, inactivating enzymatic proteins. Chlorine can penetrate the wounds and at high concentrations can produce a toxic effect, even can cause the death of the plant tissue by necrosis.

According to Druat (1997), when the tissues are damaged phenolic compounds are released, which manifest with a blackening of the culture medium around the explant and that can extend to the whole medium, causing damage to the explant growth and death. By adding activated carbon (1 to 3 g L^{-1}) to the culture medium it is possible to remove phenolic compounds and inhibitory or toxic substances from the culture medium which are produced during autoclaving, avoiding or decreasing the deterioration of the explant.

As a part of the bioassays, good results were obtained by adding 1 g L^{-1} of activated carbon and 1 g L^{-1} of polyvinylpyrrolidone (PVP) to the culture medium, results that coincide with that obtained by Senula and Keller (2000). It was found that the addition of citric acid (0.3 g L^{-1}) by submerging the explants at the end of the disinfection process is

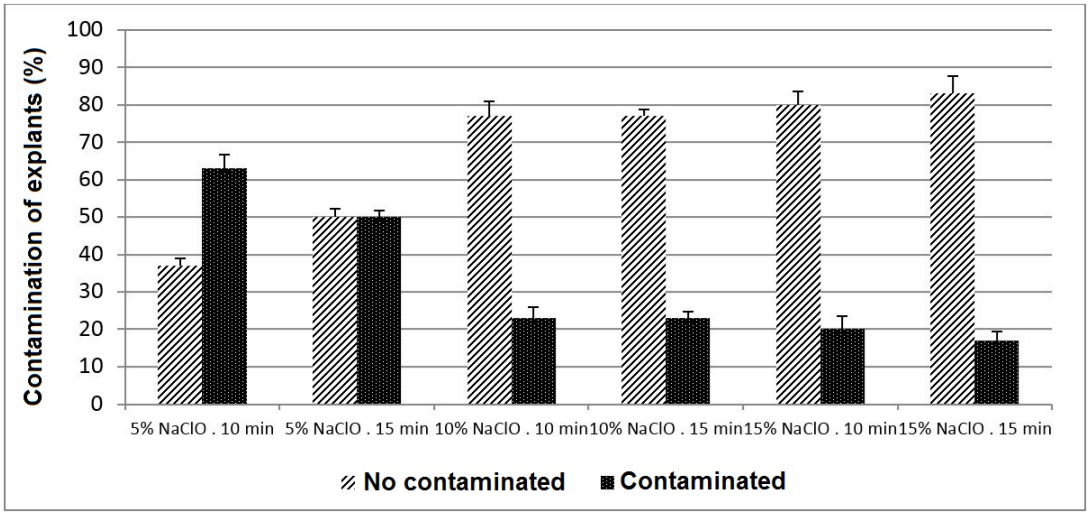

Figure 1. Percentage of contaminated explants of Emilia apple according to each disinfection treatment

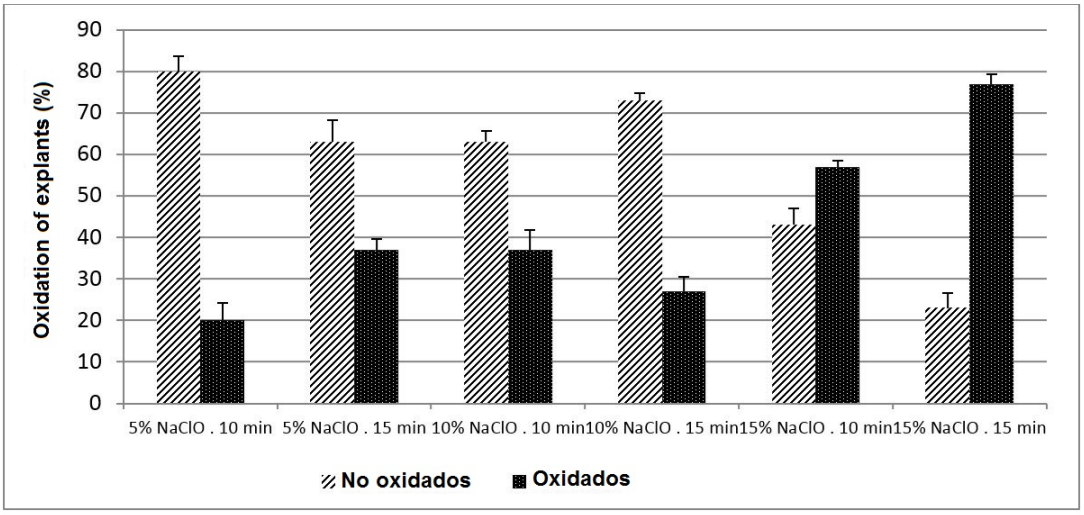

Figure 2. Percentages of oxidized apple explants Emilia

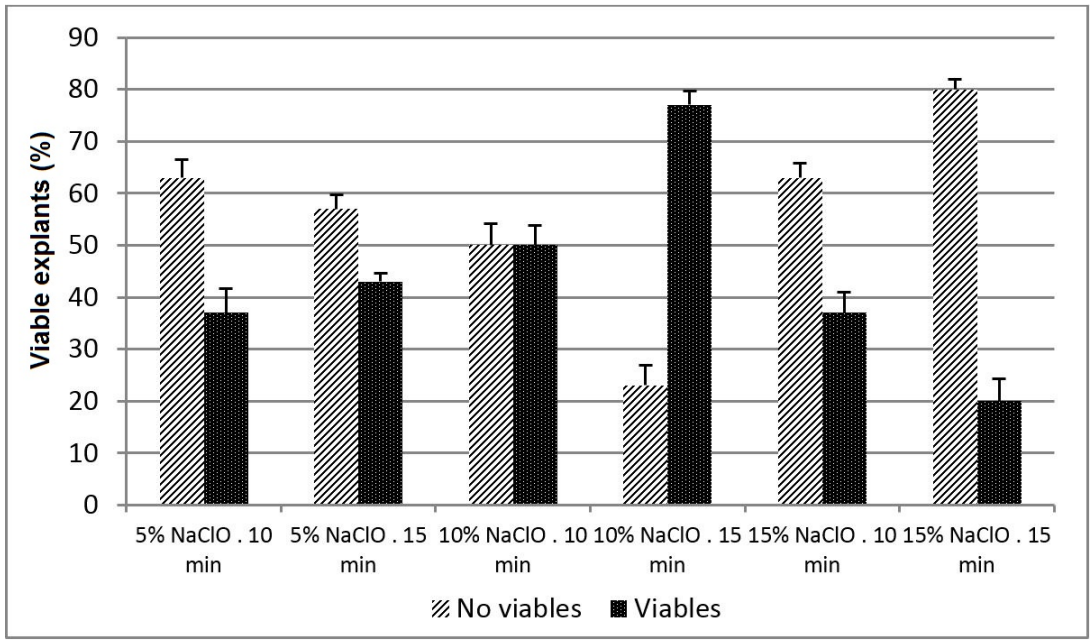

Figure 3. Percentages of viable apple explants

conducive to inhibition of the oxidation effect.

2.3 Percentage of viability

The viability of the explants was determined by the two factors: exposure time and sodium hypochlorite concentration. The treatment t4 (10% NaClO x 15 minutes) allowed to obtain 77% of viable explants, being the highest one (Figure 3). The number of contaminated explants is then reduced considerably with the increase in chlorine concentration; Although this was favorable, we also considered the number of oxidized explants, which increased as the sodium hypochlorite concentrations were increased.

Thus, it was determined that the treatment of 10% of sodium hypochlorite for 15 minutes as the best treatment in disinfection, since it allows to obtain a moderate percentage of contamination (23%), oxidation (27%) and the highest percentage of viable explants (77%). Results that are in fact similar to those reported by Engelman (2011), who obtained a range of 70 to 100% of viable explants with similar disinfection protocols; in such a way that, in practice and as part of the rescue of apple germplasm of var. Emilia, the best treatment is not one that presents the least contaminated explants, but the one that obtains the most viable explants.

2.4 Days to the shoots

In Table 2, for Variance Analysis (ADEVA) for this variable, we found highly significant differences in BAP concentrations and for each of the orthogonal polynomials. The overall average was 16.12 days and the coefficient of variation was 11.01%.

Of the orthogonal polynomials, the linear tendency is emphasized, since as the concentration of BAP increases, so does the time of sprouting and, therefore, the delay in the emergence of buds. These results are probably due to the fact that this apple variety has a high accumulation of endogenous hormones, so that the control treatment presented the best results; in accordance with Itoiz (2000), who states that cytokinins per se induce the formation and budding of adventitious buds and the development of axillary buds, so the exogenous requirements of hormones depend on the endogenous levels of each plant.

Similarly, Table 3 shows that there are seven statistical significance ranges for this variable, presenting the best responses in the treatments without

Table 2. Analysis of Variance for days at sprouting, number of shoots and length of shoots of Emilia explants

Sources of variation	gl	Medium squares		
		Days of shoots	Number of shoots	Length of shoots (cm)
Total	47			
Culture media	1	0,76ns	0,00ns	0,11ns
BAP dose	3	795,42**	1,89**	3,90**
Lineal	1	2100,42**	0,27ns	1,57**
Quadratic	1	252,08**	3,00**	6,92**
Cubic	1	33,75**	2,40**	3,21**
Culture media * BAP dose	3	0,75ns	0,00ns	0,01ns
Error	40	3,15	0,28	0,03
Average		16,12	2	3,13
C.V. %		11,01	26,45	5,53

DHS (ns: not significant; *: significant; **: highly significant)

Table 3. Values of physiological variables in the Emilia apple germplasm rescue study

Code	Description	Days of shoots	Number of shoots	Length of shoots (cm)
m1	MSM	15,99 ± 0,08 b	1,75 ± 0,03 a	3,19 ± 0,19 b
m2	CHU	16,25 ± 0,12 a	1,75 ± 0,01 a	3,59 ± 0,23 a
b0	0 ppm of BAP	9,17 ± 1,02 d	1,50 ± 0,02 c	5,85 ± 0,66 a
b1	0,5 ppm of BAP	12,00 ± 1,36 c	1,67 ± 0,06 b	3,55 ± 0,29 b
b2	1,0 ppm of BAP	15,67 ± 1,51 b	2,33 ± 2,01 a	3,06 ± 0,98 c
b3	1,5 ppm of BAP	27,67 ± 1,74 a	1,50 ± 0,03 c	1,07 ± 0,13 d
t1	CHU + 0 ppm of BAP	9,00 ± 2,48 g	1,50 ± 0,16 c	3,29 ± 1,02 d
t2	MSM + 0 ppm of BAP	9,33 ± 3,19 f	1,50 ± 0,19 c	2,84 ± 0,96 e
t3	CHU + 0,5 ppm of BAP	12,00 ± 0,57 e	1,67 ± 1,24 b	3,79 ± 0,12 c
t4	MSM + 0,5 ppm of BAP	12,00 ± 0,71 e	1,67 ± 1,45 b	3,33 ± 0,78 d
t5	CHU + 1 ppm of BAP	16,00 ± 4,07 c	2,33 ± 2,57 a	6,20 ± 1,55 a
t6	MSM + 1 ppm of BAP	15,33 ± 3,29 d	2,33 ± 2,79 a	5,51 ± 1,35 b
t7	CHU + 1,5 ppm of BAP	28,00 ± 5,72 a	1,50 ± 0,27 c	1,06 ± 0,02 f
t8	MSM + 1,5 ppm of BAP	27,33 ± 4,93 b	1,50 ± 0,35 c	1,07 ± 0,09 f

Averages with different letters indicate significant difference according to Tukey's test (P <0.05)

BAP application, which corroborates previous studies on the totipotentiality of the cells present in the endogenous hormones (Verdeil et al., 2007).

2.5 Number of shoots

In the ADEVA (Table 2) we found highly significant differences for BAP concentrations and for quadratic and cubic orthogonal polynomials. The overall average was 2 sprouts per explant and the coefficient of variation was 26.45%. Of the orthogonal polynomials the quadratic tendency is emphasized, where the effect of the hormonal compounds is reduced by the excess in its concentration.

For this variable, three ranges of statistical significance were presented (Table 3). The best responses were obtained in the treatments with application of 1 ppm BAP, with similar average for each one (2.33 explant explants-1); These results are acceptable if we compare the multiplication index by axillary buds for other apple cultivars that is in the 1: 3 ratio, as is the case with the clonal pattern MM 106 (Forsline et al., 2003).

The number of shoots is influenced by the auxin - cytokinin ratio. Numerous reports indicate that other factors are also involved in the organogenic pathway, including other growth regulators such as gibberellins (which suppress initiation of shoots or roots) and endogenous ethylene (which blocks the initiation of organogenesis but promotes growth and differentiation of preexisting roots or primordia buds (Towill et al., 2004).

2.6 Length of the shoots

In the ADEVA (Table 2) highly significant differences were obtained for culture media and BAP concentrations. The average was 3.13 cm shoot length and the coefficient of variation was 5.53%. Of the orthogonal polynomials the quadratic tendency is emphasized, where the intermediate concentrations of BAP would provide greater length of the shoots.

The best response was presented in the treatment CHU + 1 ppm BAP with an average of 6.20 cm (Table 3); these results coincide with the micropropagation project of the M9 Jork (J9) grafted apple tree, developed by Itoiz (2000), where they obtain a longer shoots length using 1 ppm of BAP. Huang et al. (1994) mention in their micropropagation study with Acacia mearnsii that higher levels of BAP promoted greater elongation of multiple shoots, the intermediate levels of this hormone in our study demonstrate adequate elongation of tissues, especially meristematic ones.

For the rooting experiments, different concentrations of IBA (0.0 ppm, 0.5 ppm, 1.0 ppm, 1.5 ppm) were evaluated, incorporating a 0.1 ppm ANA + 1 ppm Riboflavin base. The best results were obtained with the treatment (0.5 ppm of IBA), which determined 50% of rooted explants; in the other treatments, significant losses were present due to the presence of callus, bacterial contamination and oxidation.

3 Conclusions

In vivo conservation has its limitations. In vitro techniques represent a mandatory step in the use of genetic resources of fruit trees. Yet they may show considerable limitations such as genetic instability and duration of tissue storage. The cellular tissues in the Emilia apple show high perception for susceptibility to microbial contaminants and to the oxidation of tissues, which makes it difficult to multiply by in vitro culture; however there are alternatives that we have tested as the Activated Carbon antioxidant at a dosage of 1 g L^{-1} in combination with Polyvinyl pyrrolidone 1g L^{-1}, which helps reduce oxidation in the establishment phase.

The best response for disinfection of explants of nodal segments of var. Emilia to work in vitro culture technique was treatment with 10% Sodium Hypochlorite for 15 minutes; subsequently implemented in the culture media MSM and CHU, because it allows a high percentage of viability (77%) and homogeneous reduction of oxidation and contamination (23%) in both cases.

The treatments based on CHU + 0 ppm of BAP and MSM + 0 ppm of BAP presented the best responses in the variables days at bud and number of buds per explant. However, the best results regarding the variable length of the shoot were presented for treatments with both means of disinfection and 1 ppm of BAP in CHU culture medium. It was possible to determine that high concentrations of BAP inhibit spontaneous physiological responses in explants of the Emilia apple, especially in the initial stages where there is greater production of intrinsic hormonal effects.

It is important to emphasize the importance of in situ conservation, which seeks the stability of native biotypes and may favor the maintenance or reco-

very of viable populations of species in their natural habitats.

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