Olive tree *in vitro* establishment under different culture media and explant collection periods

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ABSTRACT

In the species *Olea europaea* L. the tissue culture techniques have not been sufficiently studied by the lack of efficient establishment of protocols for various cultivars. The objective of this work was to evaluate the effect of different culture media and sampling times of explants on *in vitro* establishment of six cultivars of olive (Ascolano 315, Leccino, Maria da Fé, Coratina, Arbequina and Frantoio). The work was divided into two experiments conducted in the Fruit Tree Propagation Laboratory, Crop Science Department, Faculty of Agronomy Eliseu Maciel, Federal University of Pelotas, Brazil. Stock plants of the olive cultivars maintained in semi- hydroponic system were used. In the first experiment, nodal segments were used to establish *in vitro* culture medium MO and WPM, consisting of salts and vitamins. In the second experiment four seasons to obtain the explants were evaluate (autumn, winter, spring and summer). The WPM medium promoted greater establishment of olive explants. There is a higher phenolic oxidation in olive explants collected in winter. Spring is indicated for collecting explants, therefore favors the *in vitro* cultivar Maria da Fé, while Ascolano 315 and Arbequina cultivars have a higher rate of *in vitro* establishment by collecting explants over the year.

Key words: *Olea europaea*, micropropagation, phenolic oxidation, seasons.

INTRODUCTION

The olive tree (*Olea europaea* L.) belongs to the *Oleaceae* family and has great economic importance to Mediterranean countries. According to Teramoto et al. (2013), Brazil is the world's second olives importer and, in the last decade, Brazilian table olives imports more than doubled. However, commercial cultivation in Brazil is still a recent and expanding agricultural activity (Oliveira et al., 2009) which aims at reducing by 30% the importation of olive oils. According to Jorge (2013), the most appropriate regions for olive trees production in the gaucho soil are those located in the Campanha do Rio Grande do Sul region, next to Uruguay, although it is likely that, in this state, production has been developed in the Bagé, Dom Pedrito and Uruguaiana region.

The increasing olive tree's growing trend in the country has generated a demand for seedlings to supply the market, requiring research and new technical information on the production of these seedlings (Conab 2009). According to Coutinho et al. (2015), vegetative propagation is the most viable technique for the development of olive tree seedlings, thus maintaining the genetic characteristics of mother plants, uniformity, reduced size and production precocity. Therefore, studies on the commercial propagation of this species has become essential, including micropropagation. However, to develop micropropagation protocols, it is necessary first to establish it *in vitro*.

Tissue culture applications remain unexplored in olive trees due to the lack of efficient establishment protocols for most cultivars. One of the problems faced by this species is related mainly to *in vitro* oxidation due to phenolic compounds release. These compounds, according to Taiz and Zeiger (2013) have a variety of functions in plants, some act as defense compounds against pathogens and herbivores, and others as attraction to pollinators.

Explants *in vitro* growth is also related to culture medium formulation and may show response variations for different cultivars tissues. Therefore, many media are being tested such as MO salts and vitamins (Rugini 1984), MS salt and vitamins (Murashige and Skoog 1962) and WPM salts and vitamins, the latter developed for woody plants in general (Lloyd and Mccown 1980). Rugini (1984) proposed the MO medium for the regeneration of some olive tree cultivars.

Taking into account that the availability of healthy olive tree seedlings is a determining factor for the development of commercial orchards, the production of micropropagated seedlings may be an alternative for the production of certified seedlings. With the objective to develop protocols for the in vitro establishment of olive trees, the objective of the present work was to evaluate the effect of explants collection periods in culture media (WPM and MO), on the establishment of olive tree cultivars.

MATERIALS AND METHODS

Experiments were conducted at the Department of Plant Science Fruit Plants Propagation Laboratory from Faculdade de Agronomia Eliseu Maciel, Universidade federal de Pelotas, RS. For the olive tree *in vitro*

establishment, four-year-old mother plants from the Germoplasm Active Bank of UFPel, were kept in 90L vases under a hydroponic system, irrigated with nutritive solution and formulated by Schuch and Peil (2012), according to the needs of the culture. To reduce *in vitro* contamination, mother plants were pulverized every two days, with the minimum of three applications, with Kasumin® (bactericide) and Cercobin® (fungicide), at the dosages of 3 $ml.L^{-1}$ and 0.7 $g.L^{-1}$, respectively.

New olive tree shoots, with approximately 2 cm and two buds, had their leaves removed at collection. First, explants were disinfected with alcohol at 70%, under agitation, for 1 minute. Then they were immersed in sodium hypochlorite at the concentration of 2.5% of active chlorine, adding two drops of Tween 20, for 15 minutes, in contact with the explants, under agitation. Next, the disinfected material was washed three times using autoclaved distilled water and sterilized in a laminar flow chamber, for posterior explants isolation.

In the first experiment, the in vitro establishment of olive tree cultivars was assessed under different culture media. The experiment included a 6x2 (cultivars and culture media) factorial design. Treatment variation sources were cultivars Ascolano 315, Leccino, Maria da Fé, Coratina, Arbequina e Frantoio and the culture media: WPM and MO.

The experimental design was entirely randomized, with 12 treatments and four replications, using 12 tubes per replication, with one explant per test tube. Nodal segments of approximately 2 cm were used for the *in vitro* establishment. Tested culture medium included MO salt and vitamins (Rugini 1984) and WPM salts and vitamins (Lloyd and Mccown 1980), plus 2 $mg.L^{-1}$ BAP with 100 $mg.L^{-1}$ of myoinositol, 30 $g.L^{-1}$ of sucrose, with the pH adjusted to 6.0 before the inclusion of agar at the concentration of 6 $g.L^{-1}$ and, later, autoclaved at 121ºC and 1.5 atm for 20 minutes. Test tubes (150 x 20 mm) contained 7 ml of culture medium.

After inoculation, explants were kept in the dark for seven days to reduce phenolic oxidation. Next, they were transferred to a growth room with photoperiod of 16 hours of light and 8 hours of darkness, with radiation of 27$\mu mol.m^{-2}s^{-1}$ and temperature of 25±2ºC. The experiment evaluated collection periods at the *in vitro* establishment if olive tree cultivars.

The experiment adopted a 6x4 factorial design (cultivars and collection periods). The following cultivars were the treatments variation sources: Ascolano 315, Leccino, Maria da Fé, Coratina, Arbequina and Frantoio and the following collection periods: (Fall, Winter, Spring of 2012 and Summer of 2013). Experimental design was entirely randomized, with 24 treatments and 4 replications, using 12 tubes, with an explant per replication. The culture medium for the *in vitro* establishment of nodal segments included WPM salts and vitamins (Lloyd and Mccown 1980) plus 2 $mg.L^{-1}$ BAP.

In both experiments, evaluations took place at 7, 14, 21 and 28 days of cultivation to assess bacterial contamination, fungal contamination and oxidized explants percentages. Flasks that showed contamination and/or oxidation were eliminated after registration. At 45 days of cultivation, the material was evaluated in regards to survival percentage, indicated by the green color of the nodal segment, and establishment percentage, determined by the development of leaf primordia and the presence of shoots (Figure 1).

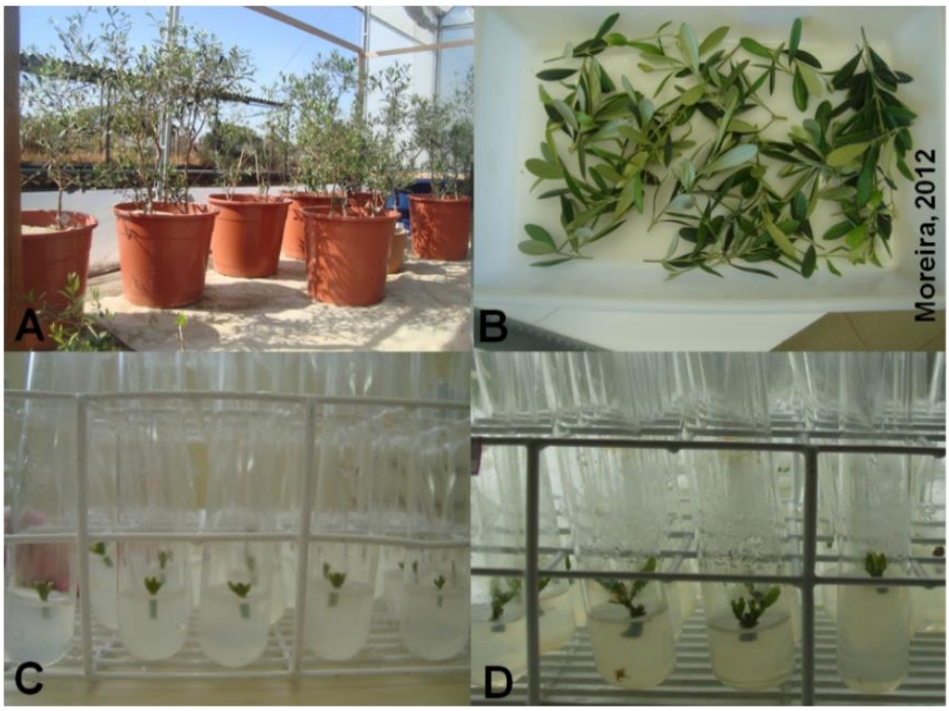


Figure 1. Stock plants of olive (A), material collection (B), inoculated explants *in vitro* (C), and explants established in a WPM culture mean at 45 days of cultivation, 2012 (D). Pelotas, RS, Brazil. 2014.

Data were submitted to an analysis of variance by the F test and means compared by the Tukey test at 5% of probability of variables analyzed sequentially (at different cultivation days). Regressions among established variables were significant whenever p≤5% by the WinStat statistical program (Machado and Conceição 2010). Data in percentages were transformed in the arc sine of $\sqrt{\frac{X}{100}}$, were x is the percentage.

RESULTS AND DISCUSSION

Results showed an interaction between cultivars and the culture media in the first experiment. Higher oxidation percentages were found for the MO culture medium for most cultivars, probably due to its highest salt concentrations. Cultivar Frantoio showed no significant difference for this factor (Table 1). These data differ for those presented by Donini et al. (2008b) on olive tree *in vitro* establishment, in which the authors obtained a low oxidation percentage for the MS, MO and WPM media. However, this same work shows high rates of fungal contamination for the three tested media.

According to Table 1, cultivar Maria da Fé showed the highest fungal contamination for the WPM medium. By studying disinfestation and culture medium in the *in vitro* establishment of *Liquidambar styraciflua* nodal segments, Brondani et al. (2010) obtained reduced fungal contamination values (14%) in a WPM medium. They immersed explants in NaOCl at 5% (v/v) (approximately 2.5% of active chlorine), for 10 minutes and in a benomyl-based solution for 40 minutes, at 1% (p/v), as an active ingredient. In an *in vitro* establishment experiment with cultivar Koroneiki, Donini et al. (2008a) obtained 26.3% of contaminated explants in a WPM culture medium.

Results showed low bacterial contamination and no significant difference between cultivars Leccino, Coratina and Frantoio for the tested media (Table 1). Dias et al. (2013), working on the *in vitro* establishment of pomegranate tree (*Punica granatum* L.), also found low bacterial contamination (2.35% of explants) at 30 days of *in vitro* cultivation. In the present study, cultivar Ascolano 315 showed the highest bacterial contamination percentage for the WPM medium (Table 1). However, this percentage is lower than that found by Palu et al. (2011), who obtained 39.14% of contamination by bacteria in fig tree apical buds, using cephalothin sodium antibiotics 150 $mg.L^{-1}$ in a MS culture medium.

Table 1. Percentage of oxidation, fungal contamination and bacterial contamination in olive tree explants established *in vitro*, in MO and WPM culture media, 2012. Pelotas, RS, Brazil. 2014.

Cultivars	Oxidation (%)	
	Media	
	MO	WPM
Ascolano 315	43.22 aC[1]	14.06 bD
Leccino	57.29 aB	29.68 bBC
Maria da Fé	55.72 aB	25.52 bCD
Coratina	76.04 aA	39.58 bB
Arbequina	55.72 aB	22.39 bCD
Frantoio	71.35 aA	66.66 aA
	Fungal contamination (%)	
Ascolano 315	27.60 aA	24.47 aB
Leccino	25.00 aA	24.47 aB
Maria da Fé	18.75 bAB	48.95 aA
Coratina	10.41 aBC	10.93 aC
Arbequina	12.49 aBC	7.29 aC
Frantoio	12.49 aC	7.29 aC
	Bacterial contamination (%)	
Ascolano 315	4.16 bA	13.54 aA
Leccino	2.60 aA	1.56 aCD
Maria da Fé	0.00 bA	3.64 aCD
Coratina	4.16 aA	5.20 aBC
Arbequina	1.04 bA	8.33 aB
Frantoio	2.08 aA	0.00 aD

[1]Uppercase letters compare columns and lower case compare lines.

At 45 days of *in vitro* cultivation, there was a significant difference between the tested media, making the highest percentage of explants established in a WPM culture mean more evident, according to Table 2.

Table 2. Percentage of *in vitro* established and survivor olive tree explants in MO and WPM culture media, at 45 days of cultivation, 2012 Pelotas, RS, Brazil. 2014.

Cultivars	Establishment (%)	
	Media	
	MO	WPM
Ascolano 315	2.08 bA[1]	31.24 aAB
Leccino	0.00 bA	27.08 aAB
Maria da Fé	8.33 aA	16.67 aBC
Coratina	0.00 bA	14.58 aBC
Arbequina	0.00 bA	37.50 aA
Frantoio	0.00 aA	8.33 aC
	Survivors (%)	
Ascolano 315	0.00 a	0.00 aB
Leccino	0.00 b	6.25 aAB
Maria da Fé	0.00 a	0.00 aB
Coratina	0.00 b	12.5 aA
Arbequina	0.00 a	4.16 aB
Frantoio	0.00 a	4.16 aB

[1]Uppercase letters compare columns and lowercase compare lines.

Cultivars Arbequina and Ascolano 315 showed the highest means in the WPM medium. These data agree with those found by Donini et al. (2008b), who observed better shoot means for the establishment of the 'Arbequina' olive tree in a WPM medium.

As for the survival evaluated at 45 days of cultivation, there was no surviving explant in the MO culture medium (Table 2), which is related to the high rate of oxidized explants. WPM showed 12.5% of surviving explants for cultivar Coratina. This result differs from that found by Costa et al. (2007), who obtained 37.50 % of rosemary pepper (*Lippia sidoides* Cham.) surviving explants in the WPM culture medium. However, Bassan et al. (2006) verified a highest cassia fistula (*Peltophorum dubium* (Spreng.) survival and establishment rate in a MS medium than those maintained in WPM.

The second experiment showed significant difference for the phenolic oxidation variable, in the Fall, for cultivars Frantoio e Leccino, which showed 79 and 39% of oxidized explants, respectively,at 28 days of *in vitro* cultivation (Figure 2A). Donini et al. (2008a), studying the *in vitro* establishment of olive tree cultivars under different light quality, verified that cultivar Frantoio showed the highest phenolic oxidation rate at 21 and 2 days of *in vitro* cultivation.

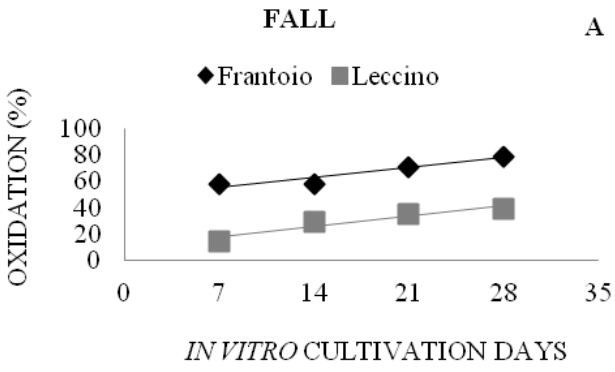
Cultivar Arbequina showed the highest oxidation percentage at 21 days of *in vitro* cultivation in winter in relation to the other tested cultivars (Figure 2B). Dias et al. (2013) obtained 82.37% of oxidized explants for the in vitro establishment of pomegranate trees.

In spring, cultivars Maria da Fé and Frantoio showed no significant difference in regards to phenolic oxidation and cultivar Leccino showed the lowest percentage (41%) in this period (Figure 2C). Scherer-Salvaro et al. (2009) observed a high oxidation percentage in java citronella explants (*Cymbopogon winterianus* Jowitt) collected in winter and spring and established *in vitro*.

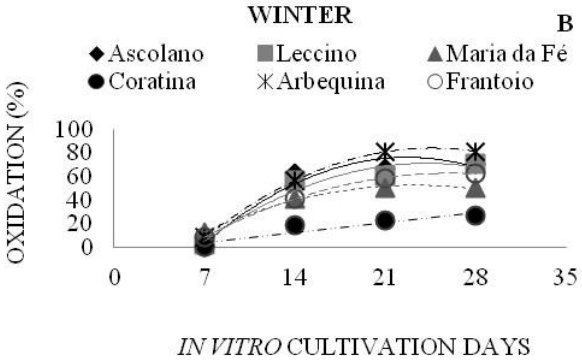
Figure 2D shows the increasing linear response from explants oxidation in function of an increase in number of cultivation days in the five cultivars tested in summer, with no significance only for the Maria da Fé cultivar. Cultivars Leccino and Coratina showed the highest oxidation percentage at 28 days, which could be related to internal and external factors that control the production of phenolic compounds such as hormones and light

In regards to bacterial contamination, there was an increase throughout the *in vitro* establishment for explants from the Coratina cultivar collected in the Fall (Figure 3A) and Maria da Fé cultivar collected in the Winter (Figure 3B), corresponding to 10 and 20% of the contaminated explants at 28 days of cultivation. During the *in vitro* establishment of the blueberry tree cultivars Bluecrop, Misty and Duke, Pelizza et al. (2013) obtained, respectively, 25.02%, 20.03% and 27.29% of bacterial contamination.

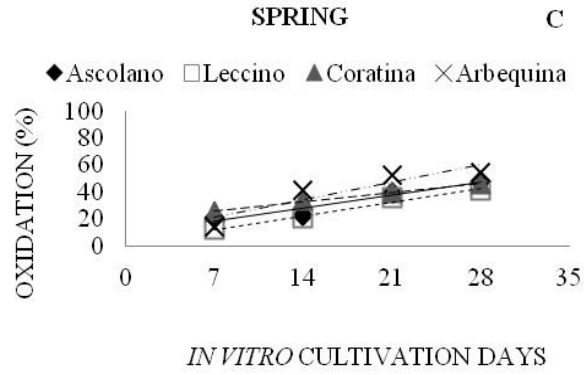
The analysis of variance showed no significant effect for fungal contamination in different olive tree cultivars tested for the four seasons of the year. These results differ from those found by Donini et al. (2008a), who obtained 26.3% of contamination in the *in vitro* establishment of the Koroneiki olive tree cultivar.



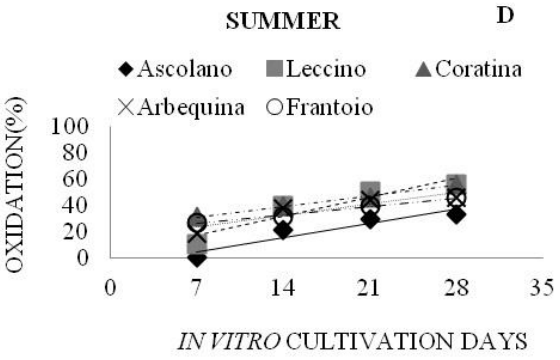
Frantoio: y= 7.50x + 55.42 R^2 = 0,9
Leccino: y= 8.12x + 17.50 R^2 = 0,92 .



Ascolano: y= -14.58 x2 + 65.42x + 2.92 R^2= 0.95
Leccino: y= -10.94x2+53.85x + 4.90 R^2= 0.94
Maria da Fé: y= -7.30x2 + 33.95x + 13.12 R^2= 0.99
Coratina: y= 8.54x + 4.38 R^2= 0.85
Arbequina: y= -11.98 x2 + 60.31x + 8.22 R^2= 0.99
Frantoio: y= -7.39x2 + 39.80x + 8.54 R^2= 0.99

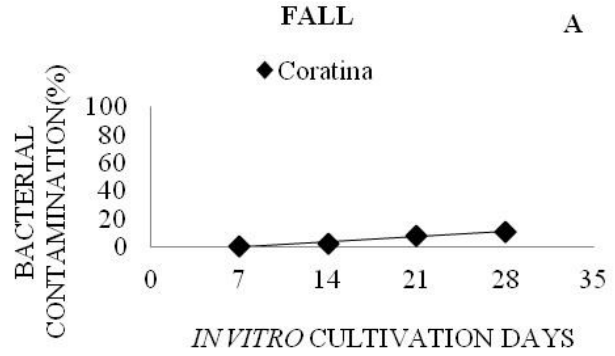


Ascolano y= 9.80x + 18.12 R^2= 0.93
Leccino y= 10.20x + 12.30 R^2= 0.98
Coratina y= 6.87x + 25.62 R^2= 0.99
Arbequina y= 12.91x + 21.25 R^2= 0.83

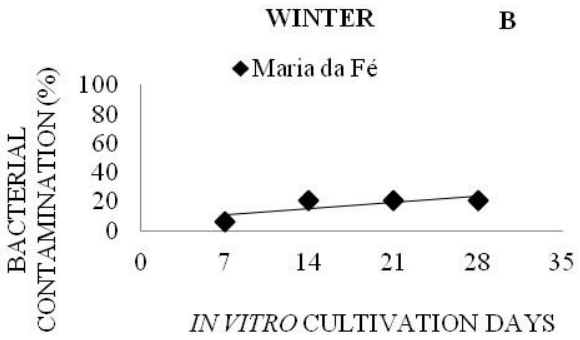


Ascolano y= 9.80x + 18.12 R^2= 0.93
Leccino y= 10.20x + 12.30 R^2= 0.98
Coratina y= 6.87x + 25.62 R^2= 0.99
Arbequina y= 12.91x + 21.25 R^2= 0.83

Figure 2. Percentage of oxidized olive tree explants collected in the Fall (A), Winter (B), Spring (C) and Summer (D) and established *in vitro*, 2013. Pelotas, RS, Brazil. 2014.



Coratina y= 3.74x – 0.42 R^2= 0.95



Maria da Fé y= 4.37x + 10.62 R^2= 0.60

Figure 3. Percentage of olive tree explants contaminated by bacteria, collected in the Fall (A) and Winter (B) and established *in vitro*, 2012. Pelotas, RS, Brazil. 2014.

As for the olive tree explants oxidation, survival and establishment percentages, there was no interaction between the tested cultivars and collection periods. The highest oxidation percentages were shown by cultivars Frantoio in the Fall and Arbequina in the Winter, differing significantly from the collection realized in the Summer (Table 3). Costa et al. (2007) observed a strong difference in regards to different periods of the year for the establishment of *Lippia sidoides* Cham, considering that the dry period, when the relative air humidity was lower, favored the establishment; however, during the rainy season, there was greater incidence of microorganisms, mainly endogenous bacteria, plus oxidation.

Table 3. Percentage of oxidized olive tree explants established and survivors, collected in different seasons of the year, at 45 days of *in vitro* cultivation, 2012. Pelotas, RS, Brazil. 2014.

	Oxidation (%)			
	Fall	Winter	Spring	Summer
Ascolano 315	25.00 b[1]	75.00 a	70.83 a	58,33 a
Leccino	39.58 b	75.00 a	72.91 a	66.67 ab
Maria da Fé	29.16 ab	50.00 a	16.66 b	50.00 a
Coratina	50.00 ab	29.16 b	77.08 a	68.75 a
Arbequina	39.58 b	83.33 a	54.16 b	45.83 b
Frantoio	83.33 a	64.58 ab	79.16 a	47.91 b
	Established (%)			
Ascolano 315	31.25 a	2.08 c	18.75 ab	4.16 bc
Leccino	27.08 a	4.16 b	16.67 ab	8.33 b
Maria da Fé	16.67 b	0.00 c	62.50 a	4.16 bc
Coratina	14.58 a	6.25 a	12.50 a	14.58 a
Arbequina	37.50 a	0.00 b	2.08 b	10.42 b
Frantoio	8.33 ab	12.5 ab	0.00 b	14.58 a
	Survivors (%)			
Ascolano 315	0.00 b	10.42 ab	10.42 ab	10.41a
Leccino	6.25 a	10.42 a	10.42 a	8.33 a
Maria da Fé	0.00 b	0.00 b	14.58 a	6.24 ab
Coratina	12.50 a	12.50 a	10.41 a	8.33 a
Arbequina	4.16 a	0.00 a	2.08 a	4.16 a
Frantoio	4.16 a	8.33 a	14.58 a	6.25 a

[1]Lowercase letters differ among them in the line by the Tukey test at 5% of probability.

CONCLUSIONS

The WPM culture medium promotes higher percentage of olive tree explants establishment.
There is higher phenolic oxidation in olive tree explants collected in winter.
Spring is recommended for cultivar Maria da Fé explants collection, while cultivars
Ascolano 315 and Arbequina show higher *in vitro* establishment rate in the fall.

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