IN VITRO CLONAL BEHAVIOR OF *OLEA EUROPAEA* L., VARIETY GALEGA VULGAR

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RESUMO

A multiplicação clonal da cultivar de oliveira Galega vulgar por estacaria semi-lenhosa, é impedida pelas baixas taxas de enraizamento, as quais, segundo alguns autores, não ultrapassam os 10%, originando assim desequilíbrios entre a procura e a oferta bem como preços de mercado mais elevados face a outras cultivares de fácil enraizamento. A micropropagação por rebentação axilar surge como uma técnica alternativa de clonagem, por originar rejuvenescimento das plantas e taxas de enraizamento *in vitro* próximas ou iguais a 100%. Utilizando estacas de árvores adultas do pomar clonal de Galega vulgar existente na ESAS, anterior e parcialmente caracterizado por outras Instituições para a) presença de vírus; b) taxas de enraizamento; c) rendimento em azeite, introduziram-se aleatoriamente 39 clones *in vitro*, existindo actualmente 15 clones *in vitro*. Sendo raras as publicações sobre estudos de diferenças clonais no comportamento *in vitro* na oliveira e noutras espécies, este trabalho apresenta os resultados obtidos nos estudos efectuados desde 2003 para 15 clones, respeitantes à 1) introdução de material vegetal; 2) controlo de bacterioses sistémicas; 3) caracterização da variação interclonal nas taxas de multiplicação, enraizamento e aclimatação.

Palavras-chave: Oliveira; Galega vulgar; micropropagação; clone; variação clonal

ABSTRACT

Clonal multiplication of olive variety Galega vulgar trough leafy steam cuttings is impeded by low rooting rates, according to some authors do not overcome 10% of success. Those situations originates imbalances between plant offer and market demand as well as higher prices of plants face others varieties. Micropropagation trough adventitious multiplication is a technical proceeding able for cloning recalcitrant woody plants. Trough rejuvenation of plants, rates of 100% in rooting is achieved. Using cuttings from adult trees of a clonal orchard of Galega vulgar in ESAS, previously and partially characterized for a) presence of virus; b) rooting rates of cuttings; c) olive oil yield, 39 clones where randomly introduced *in vitro* for an actual collection of 15 *in vitro* clones. Published papers concerning *in vitro* clonal behavior for olive or other species are rare, thus, this work presents the obtained results since 2003 for 1) introduction of clones; 2) systemic bacterioses control; 3) clonal variation in multiplication, rooting and acclimatization.

Key words: Olive; Galega vulgar variety; micropropagation; clone; clonal variation

INTRODUCTION

Galega vulgar is an olive tree variety extensively grown in Portuguese traditional orchards, where it represents more than 70% of the trees. The aromatic features of the Galega vulgar oil (Peres M.F. *et al*., 2002) and its content in tocopherols and oleic acid (Gouveia J.M. *et al*., 2003; Gouveia J.M., 2007) make it a very peculiar olive oil easily distinguishable from oils of other cultivars, with a higher content in vitamin E. The European Union produces 78.1 % of the world olive oil production and Portugal produces 1.7 %. This scenario sustains the importance of this cultivar in the global market of olive oil. Clonal propagation of Galega vulgar trees has been carried out by cuttings or grafting (Leitão F., 2004). Labor costs and the extended periods of time required to obtain the desired results have limited a wider application of grafting to Galega vulgar commercial propagation. On the other hand, propagation through leafy steam cuttings under mist is highly conditioned by the low rooting rates occurring in this variety (Leitão F., 2004). Considering these drawbacks, micropropagation emerges

as an effective alternative way for Galega vulgar cloning. Effective protocols for commercial micropropagation through axillary shoot proliferation have been used in many olive tree cultivars (Rugini and Pesce, 2006; Garcia-Ferriz L. *et al*., 2002; Zuccherelli G. and Zuccherelli S., 2002) even considering some varieties very recalcitrant to the *in vitro* culture (Zuccherelli G. and Zuccherelli S., 2002). In the case of Galega vulgar, a protocol for the *in vitro* cloning was established (Jacob A.P., 2001; Peixe A**.,** et al., 2007) overcoming rooting problems.

In vitro culture introduction of thirty nine clones of Galega vulgar, collected from adult material in field or in green house resulted on thirteen clones actually in routine production. Entophytic bacteria contamination and recalcitrance for *in vitro* culture strongly conditioned introduction and methodologies for individualization of introduction of shoots needed to be accessed. Apical dominance limits secondary axillary shoots production in Galega vulgar, as referred by Gyves *et al.,* 2008 for other olive cultivars, and the procedure for multiplication needs to be performed trough the cutting of the formed plant into uninodal cuttings. *In vitro* rooting is performed dipping individualized explants bases in an auxin sterilized solution, in a process called 'pulse'. Distinct multiplication and rooting rates were found within clones. A preliminary evaluation of Galega vulgar clones in field conditions has showed distinct behavior in virus content, rooting rates and olive oil fat content. Similarly, distinct behavior is observed *in vitro*. Plant *in vitro* culture literature is sparse reporting studies in *in vitro* micropropagation behavior within clones, whatever the plant species. The main goal of the present study is to relate the distinct behavior of the 39 clones introduced *in vitro*, especially in what concerns introduction, multiplication, rooting and acclimatization procedures.

Abbreviations

2ip – 2-isopentenyladenine

BAP – Benzylaminopurine

COI – Conselho Oleícola Internacional

ESAS – Escola Superior Agrária de Santarém

IBA – indol-3-butyric acid

OM – Olive Culture Medium

OMm - Olive Culture Medium, multiplication formulation

OMe - Olive Culture Medium, rooting expression formulation

ppm- parts per million

Zeat – Zeatin

KCl - Potassium Chloride

MATERIALS AND METHODS

We used a standard protocol of micropropagation based on the works of Jacob AP., 2001 and Peixe A**.,** 2007. 39 clones from a germplasm collection of olive Galega *vulgar* variety were used. Herbaceous sprouts were collected from individually marked plants in field and in green house conditions. Single node segments were prepared and sterilized according to Peixe A**.,** 2007. All culture media were in accordance with the proposed by Rugini *et al*., 1984 – OM culture medium. Inoculation of uninodal cuttings were done individually in tubes containing 2 ml of OM (Olive Culture Medium) supplemented with 2.28 µM zeat (zeatin). After 30 days in culture, infected and nonreactive explants were visually detected and registered. Sprouting scions were transferred to vessels with 30 ml of OM supplemented with 30 g l^{-1} mannitol, 7 g l^{-1} commercial agar, 4.56 µM zeatin and 8.88 µM BAP (Benzylaminopurine) – OMm (Olive Culture Medium, multiplication formulation). After 30 days culture period, infected and non-reactive scions were eliminated and registered. Sprouted scions were transferred to glass vessels with 90 ml of OMm. After 30 days culture period, plantlets were sectioned into single node fragments and transferred to OMm culture medium. During 60 days and each 30 days, plantlets were cleaned of callus formation and transferred to new OMm. 90 days after first cut, plantlets are again sectioned, with apical parts with at least three nodes used for root induction and expression – OMe (Olive Culture Medium, rooting expression formulation) and basal parts sectioned in single node cuttings placed in OMm. Rooting induction was performed through 10 seconds immersion of basal part of plants into 19.682 µM filter sterilized IBA (indol-3-butyric acid) solution. Inducted plants were transferred into OMe without growth regulators supplemented with 2 g l^{-1} of activated charcoal and 30 g l^{-1} sucrose during 30 days for root induction; pH of all culture medium formulations were stabilized at

5.8 with KCl (potassium chlorate) prior to sterilization. All media components were autoclaved (121 ^{0}C, 15 psi, 20') and medium distribution was done at Flow Laminar Hoods. Cultures were maintained at growth chambers with 24/21 ^{0}C day/night, 16 hours photoperiod and 36 µmol m^{-2} s^{-1} of light intensity was provided by white fluorescent lights. Acclimatization was conducted using commercial nurseries facilities and distinct pilot conditions with control of light intensity, photoperiod, relative humidity and temperature in some of the experiments. Preliminary trials were performed to test the influence of sucrose reduction in rooting expression medium and of plants multiplied on semi-temporary immersion systems (RITA®) in acclimatization rates. For statistic treatment of data SPSS program was used, for data collected from experiments conducted in completely randomized designs. Distribution and data homogeneity analyses were verified for population variations and variance analyses were performed to investigate differences among clones or between factors.

RESULTS AND DISCUSSION

I – *In vitro* introduction

A previously and partial characterization of clones in field conditions were performed by other Portuguese institutions in the scope of several projects and are summarized in Table I for the clones introduced *in vitro* by ESAS. The most relevant analyzed characteristic is the distinct content of fat in olive oil within clones.

Table I – Characterization of clones in field conditions

Clone	Rooting rate	Fat content in oil	Virus
PSL	20	-	-
0021	61	-	-
0091	2	-	-
0102	4	-	-
0181	8	-	-
0433	11	-	-
0462	7	-	-
0501	15	-	-
1031	7	55	-

1061	10	45-50	-
1062	1	55	NI
1071	16	-	-
1081	2	-	-
1171	7	-	-
1362	11	-	-
1382	4	45-50	-
1441	1	-	-
1452	8	-	-
1461	12	-	-
1462	4	45-50	-
1482	10	50-55	-
1492	1	45-50	-
1501	13	-	-
2051	5	50-55	0
5052	3	-	0
2062	8	50-55	-
2071	5	50-55	-
2092	7	50-55	-
2131	10	50-55	-
2181	17	-	0
2211	4	55	-
2212	21	50-55	0
2221	43	45-50	-
2232	2	-	-

NI – *Nicotiana rangsdorffii*

Number of *in vitro* introduced scions, by clone, and number of unviable scions due to fungus or bacterial infections (1 month culture) or recalcitrance (after 2 months culture), are presented on Table II. With the exception of PSL clone, all the other scions were collected from potted non-productive plants, under environmental controlled conditions, without application of phyto-pharmacological products. Endophytic bacteria were present in some clones and observed many months after *in vitro*

introduction. In spite of Galega vulgar plantlets being tolerant to 90 µg mL^{-1} of rinfampicin, which reduces bacterial growth, the only way to eliminate them was continuous plant apex propagation. Clones with more than 50 uninodal cuttings introduced *in vitro*, presenting low rates of infections after more than two months of *in vitro* culture and not in routine, were considered recalcitrant for *in vitro* culture. Those plants presented reduced or null growth with abnormalities in leaves (Fig 1.). The plants in routine multiplication are presented in Fig. 1.

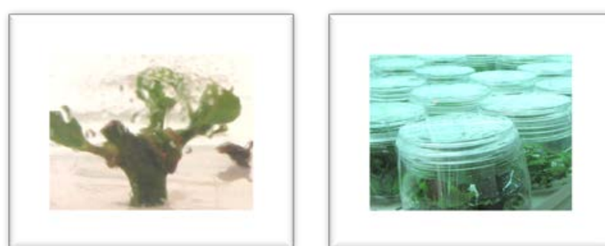

Fig. 1 – *in vitro* introduced scion of a recalcitrant clone (left) and clones in routine multiplication (right).

Table II – Number of introduced scions by clones and their behavior regarding infections and *in vitro* culture recalcitrance.

Clone	Nº of *in vitro* introduced scions	Death scions after 1 month (%)	Death scions after 2 month (%)	Routine
PSL*	6000	40	30	yes
0021	43	58	42	
0091	66	40	60	
0102	42	20	30	
0181	26	26	0	
0433	86	28	68	
0462	5	5		
0501	58	30	40	yes
1031	67	72	28	
1041	51	20	20	yes
1061	78	10	10	yes
1062	58	10	10	yes
1071	90	45	20	yes

Clone	Nº of *in vitro* introduced scions	Death scions after 1 month (%)	Death scions after 2 month (%)	Routine
1081	94	10	10	yes
1132	29	80	20	
1171	76	74	26	
1362	31	80	20	
1382	41	75	25	
1401	29	80	20	
1441	40	20	10	yes
1452	27	85	15	
1461	75	37	63	
1482	21	90	10	
1492	30	10	10	yes
1501	69	60	30	
2032	62	80	20	
2051	22	5	10	yes
5052	50	40	20	yes
2062	54	26	74	
2071	30	75	25	
2092	57	40	60	
2131	86	56	42	
2181	32	31	69	
2200	18	90	10	
2211	69	70	10	yes
2212	29	75	25	
2221	36	75	25	
2232	76	50	20	yes
1462	79	40	60	

PSL Collected cuttings from adult productive trees in field conditions.*

For PSL clone, scions were collected from adult productive trees irrigated and fertilized in field conditions. We present at Fig. 2, the *in vitro* behavior of scions collected

through the year. Note for the higher *in vitro* survival rates of scions from the summer period gathering.

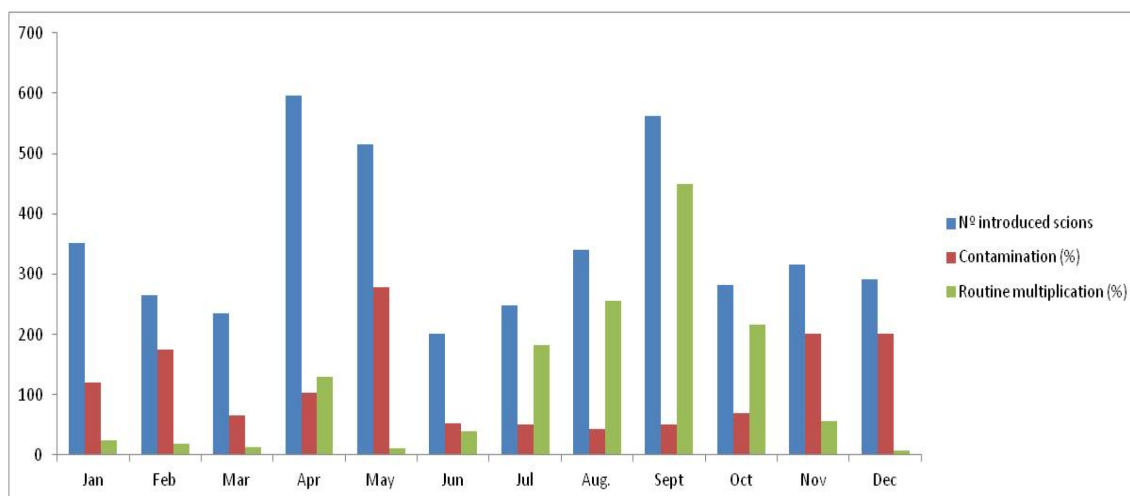

Fig. 2 – *In vitro* survival of collected scions through the year from and orchard of productive trees of PSL clone. Meteorological field conditions from 2006 in Santarém, Portugal.

II – *In vitro* multiplication

For multiplication rates we studied, for clones 1061, 1062 and 1492, the influence of 2ip (2-isopentenyladenine), BAP and zeat and some of their combinations. The results are presented on Table III and Fig. 3.

Table III – Multiplication rates on 4 weeks base for several cytokinins and their combinations.

	Mod A	Mod B	Mod C	Mod D
Clone	2 mg L^{-1}2ip	1 mg L^{-1}2ip + 2 mg L^{-1}BAP	1 mg L^{-1} Zeat + 2 mg L^{-1}BAP	1 mg L^{-1}2ip
1061	0.6	1.7	**2.5**	1.5
1062	0.4	1.1	**1.6**	0.5
1492	1.2	0.7	**2.0**	0.6

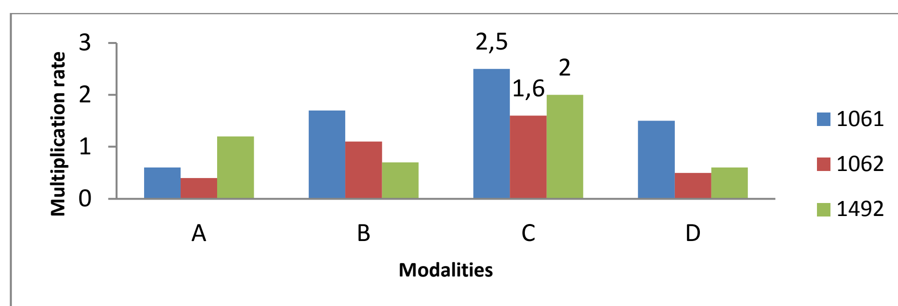

Fig. 3 – Graphic representation of multiplication rates for 3 clones at the 3 hormone conditions.

The analysis of variance shows that the 'modality' is statistically significant for the multiplication rate (Table IV) being the combination of BAP and zeat the most suitable for the highest multiplication rate in the 3 tested clones. Based on those results, *in vitro* multiplication rate on 8 weeks base were studied for 15 clones (Fig. 4). Analysis of variance showed statistically significant differences in multiplication rate among the 15 studied clones for the mentioned period (Table V), ranging from 3.5 multiplication rate for the clone 2211 and 7.0 multiplication rate for the clone 1441.

Table IV – Analysis of variance for multiplication rate on 4 weeks base (*P* ≤ 0.05).

Variation	SS	d.f.	MS	f	p
Clone	10.57	2	5.28	4.215	.0163
Modality	39.26	3	13.8	10.43	.000002
Clone X Mod.	8.37	6	1.39	1.11	0.356
Error	211.95	1.69	1.254		

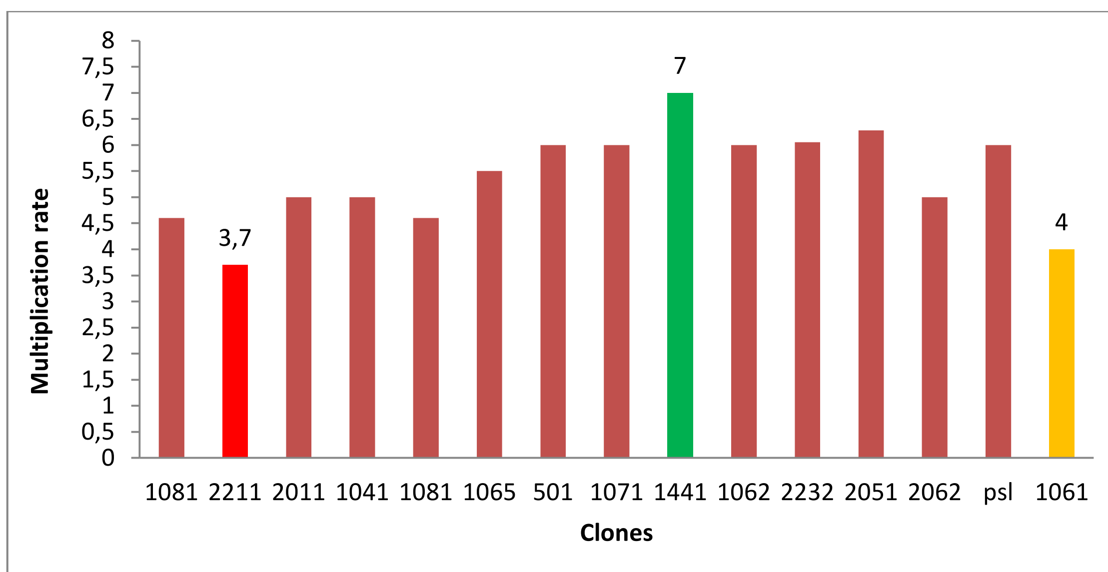

Fig. 4 – Graphic representation of multiplication rates for 15 clones at 8 weeks for OM culture medium supplemented with 2 mg L^{-1} BAP plus 1 mg L^{-1} zeat.

Table V – Analysis of variance for multiplication rate on 8 weeks base (*P* ≤ 0.05).

Variation	SS	d.f.	MS	f	p
Clone	250.527	13	19.271	8.079	.00000
Error	689.394	289	2.385		

III – *In vitro* rooting

Several experiments were performed to test *in vitro* rooting procedures alternative to 'pulse' technique (data not showed). 'Pulse' technique is a very expensive and slow method that requires the individual manipulation of plantlets for immersion in IBA sterilized solution during 10 seconds, previously to introduction into rooting expression medium. No other process showed similar efficiency to the 'pulse' technique. Based on those results, we present on Table VI the experiments testing several IBA concentrations by 'pulse' technique for each clone. The quantity of plants in multiplication limited the experimentation of all IBA formulations for some clones.

Table VI – Rooting experiments using several IBA concentrations.

Clone	IBA concentration (ppm)	nº tested plants	nº repetitions	rooting rate
PSL	2500	25	1	0
	3000	62	3	46
	3500	37	2	37
	4000	13	1	54
	4500	168	5	86
	5000	16	1	56
0021	3000	7	1	0
	4000	25	2	16
1041	3500	28	1	68
	4500	61	3	93
	5000	21	1	43
1061	3000	50	2	0
3500		146	6	75
	4500	98	4	100
	5000	80	3	77
1062	3000	23	1	43
	4000	19	1	62
	4500	129	6	86
1441	3500	63	3	83

Clone	IBA concentration (ppm)	nº tested plants	nº repetitions	rooting rate
	4500	74	3	81
	5000	12	1	0
1461	3000	7	1	34
2051	3000	11	1	18
	3500	22	1	5
	4500	21	2	58
	5000	19	1	0
2211	4500	28	1	50
	5000	19	1	28
1071	4500	74	3	85
	5000	31	2	55
1492	2500	35	2	0
	3000	9	1	0
	3500	55	3	69
	4000	57	3	15
	4500	149	3	78
	5000	23	1	4
2232	2500	14	1	0
	3000	8	1	13
	3500	37	1	30
	4000	13	1	38
	4500	11	1	18
0501	3500	18	1	56
1081	3000	50	2	62
	3500	10	1	70
	4000	98	3	26
	4500	42	2	78
2052	3000	25	1	48
	3500	51	2	59
	4000	12	1	52
	4500	91	5	42

The analysis of variance (Table VII) shows highly significant differences between the interaction 'clone X IBA ppm' for rooting percentage.

Table VII – Analysis of variance for rooting rate on 30 days base (*P* ≤ 0.05).

Variation	SS	d.f.	MS	f	p	test potency
IBA ppm	10719.01	4	2679.75	4.077	0.38	0.704
Error	5676.54	8.94	657.350			
Clone	5939.230	8	792.404	0.021	0.741	0.147
Error	8922.923	7.46	1194.76			
ppm IBA X Clone	7240.316	8	905.039	7.948	**0.000**	**1.000**
Error	6718.2	59	113.866			

Type IV Sums of Squares.

V- Acclimatization

For acclimatization of rooted plants, we tested several facilities, with or without environmental control. A private olive nursery facility was used without cooling system in the green house and using manual procedures to control environmental and moist humidity (Fig. 5). In those conditions, 1 312 plants from 5 clones where studied in different times of the year. The results are presented in Fig. 6. The analysis of variance shows no statistical differences for acclimatization survival among clones (Table VIII).

Fig. 5 – Olive clones acclimatization on a private nursery.

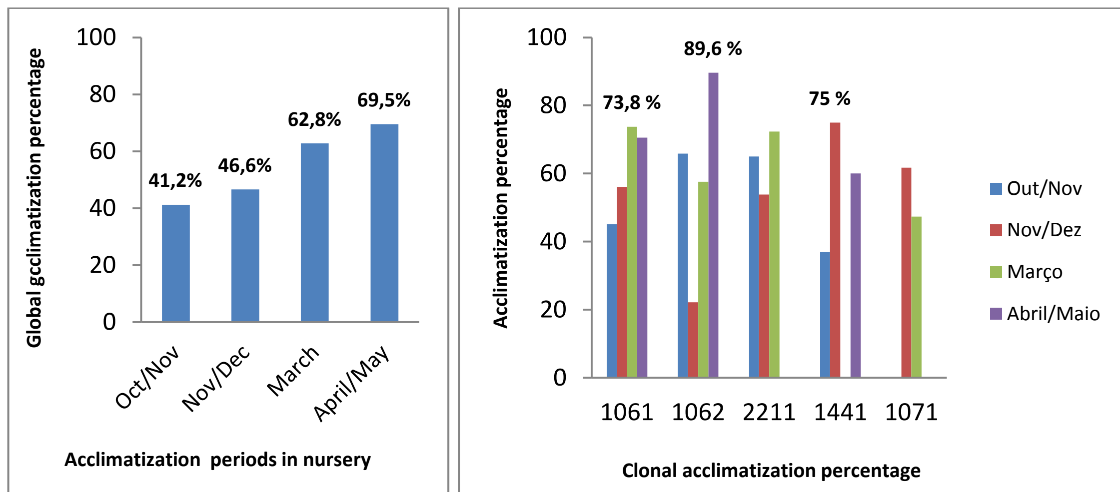

Fig. 6 – Clonal acclimatization of 1 312 plants from 5 clones (1061; 1062; 2211; 1441; 1071), in a private nursery green house, without environmental control.

Table VIII - Analysis of variance for acclimatization rate on 30 days base ($P \leq 0.05$).

Variation	SS	d.f.	MS	f	p	test potency
Period	1967.922	3	655.974	2.162	0.150	0.407
Error	336.824	10.96	303.389			
Clone	7039.114	10	703.911	2.327	0.089	0.639
Error	3404.098	11.25	302.527			
Period X Clone	3336.195	11	303.290	1.209	0.611	0.75
Error	250.800	1	250.880			

To compare acclimatization rates*, several ex vitro* conditions were tested using i) *in vitro* growth chamber, with light intensity, photoperiod and temperature control; ii) acclimatization chamber, with control of previous parameters plus relative humidity and iii) private nursery facilities, for a total of 903 tested plants for all the three systems. Survival rates of plantlets for all the tested conditions were registered and the results on acclimatization are shown in Fig. 7 and 8. In general, despite the poor control of environmental conditions in nursery, the acclimatization rates were better than in the other facilities. Notice that the air circulation in acclimatization chamber induced the dehydration of the plantlets (Fig. 7 – right).

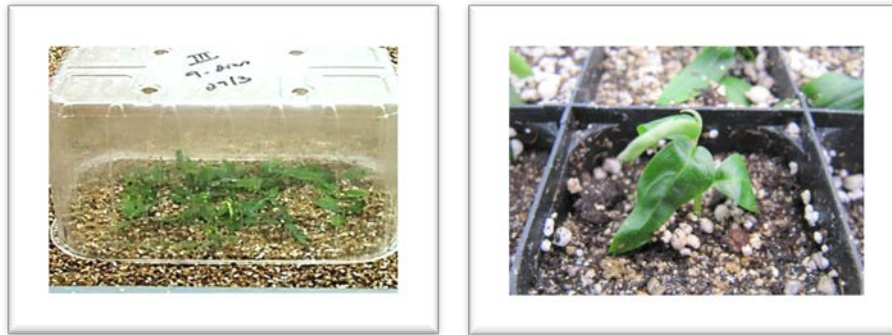

Fig. 7 – Rooted plants acclimatized in *in vitro* growth chamber (left) and in acclimatization chamber (right).

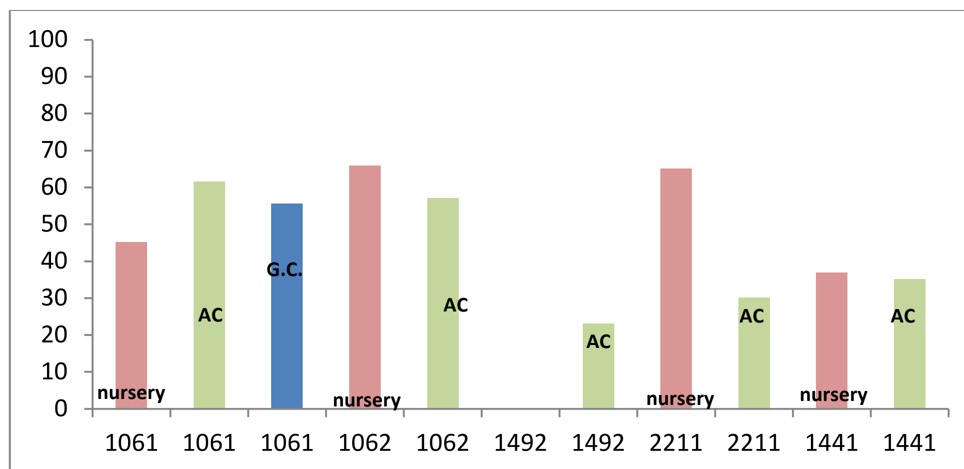

Fig. 8 – Acclimatization rates of 5 clones (1061; 1062; 1492; 2211; 1441) in distinct environmental controlled conditions, in a experiment conduced in Oct/Nov. period. (AC – Acclimatization Chamber; GC – Growth Chamber).

A preliminary experiment was performed to test plants were rooting was expressed in culture medium with sucrose reduction (50% and 0%), as indicated by bibliography for improvement of photosynthetic capacity and increase in *ex vitro* acclimatization rate. Plants multiplied in bioreactors RITA® (data not showed) were also tested. The experiment was conducted in the private nursery in April/May, period where it was observed the highest acclimatization rate. Two clones were tested. The results are expressed in Fig. 9. Plants multiplied on bioreactors showed the highest acclimatization survival and the reduction in the sucrose concentration of the rooting expression medium has not improved survival in the acclimation, compared with the normal concentration.

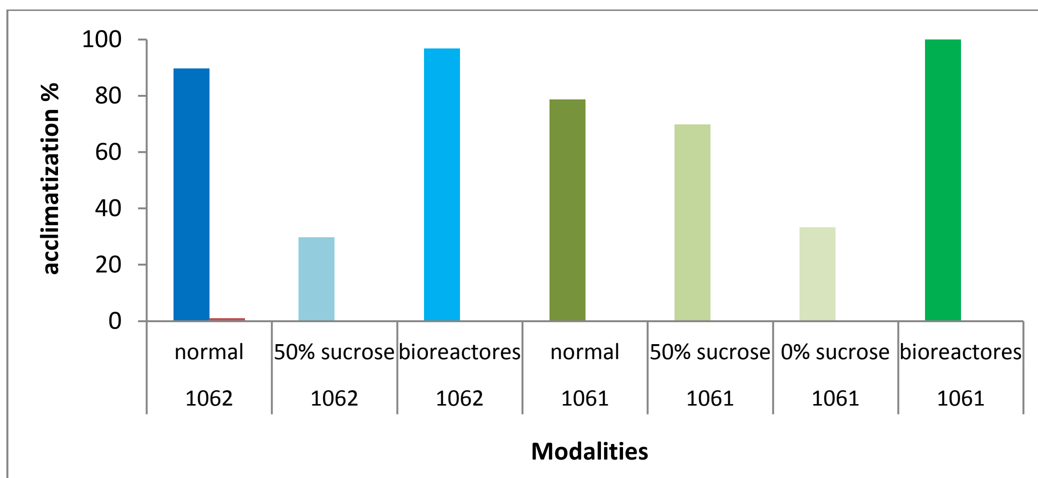

Fig. 9 – Comparison of acclimatization rates of clones 1061 and 1062, in the private nursery conditions, rooted *in vitro* with different concentration of sucrose and in bioreactors.

CONCLUSIONS

From the almost 8 000 *in vitro* introduced scions from 39 clones, only the scions of 13 clones were able to regenerate without entophytic bacteria contamination or plantlets malformations. From the presented results, we conclude that some clones are recalcitrant to *in vitro* culture or to the adapted culture medium protocol, existing variations within clones to *in vitro* culture adaptability. Collected scions from in field productive olive trees are seasonally dependent in what concerns *in vitro* viability.

Zeat and BAP combination are the most suitable cytokinines for olive *in vitro* multiplication. Statistical analyses on multiplication data of 15 clones reveal significant differences within clones for *in vitro* multiplication rates, with clone 1441 presenting the highest multiplication rate and clone 2211 the lowest.

The results for *in vitro* rooting using several IBA concentrations demonstrated the distinct rooting ability within olive Galega vulgar clones. Those clones can be grouped in three groups, namely with rooting rates of 80-100%; 60-80% and < 60% (Table VI). If we considered the traditional propagation by leafy steam cuttings commonly used for plant propagation and allocation in the market, with very low rooting rates for this variety, then, and according the *in vitro* results, a clonal procedure needs to be performed, with clones identified and individualized, and the IBA concentration adjusted for each one.

Acclimatization success depends on other conditions than the clone, namely air temperature and relative humidity control.

For the first time, a complete study is presented to verify eventual clonal differences within *in vitro* clones of olive variety Galega vulgar through the entire process of *in vitro* culture. The result show *in vitro* variability within clones.

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