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Secondary Somatic Embryogenesis for Cork Oak (Quercus Suber L.): Influence of Sugars

Naouar Benali* and Ahmed Lamarti
Laboratory of Plant Biotechnology and Plant Breeding, Department of Biology, Faculty of Science M'hannech II, BP 2121, 93002 Tetouan, Morocco, Corresponding author email id : benalinaouar@yahoo.fr

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Abstract **– This study consists in testing the secondary somatic embryogenesis technique on Moroccan cork oak (*Quercus suber L*). The experiment was conducted to test particularly the influence of different carbon sources commonly used in the induction of secondary somatic embryogenesis. We demonstrated that the sorbitol and mannitol have an inhibitory effect, whereas glucose is the most effective in terms of secondary somatic embryogenesis expression in the case of Moroccan *Quercus suber L*.**

Keywords **– Cork Oak, *Quercus Suber L*, Maamora Forest, Secondary Somatic Embryogenesis, Carbon Sources.**

I. INTRODUCTION

The cork oak (Quercus suber L.) is one of the most important species in the Mediterranean Basin in terms of ecology and cork production that contributes to the rural development in its geographical distribution sites [1], [2]. In Morocco, about 350.000 hectares are covered with this species. Genetic variability is considerable with a large range of ecotypes and various modes of growth [3].

In Morocco, the forest of Maamora occupies at present 60.000 ha, that represents 17 % of the total surface of the Moroccan cork oaks forests and 25 % of the forests surface of the Atlantic coast, and it constitutes a socioeconomic and environmental space of an extreme importance at local, regional, national and international level.

However, the growth of cork demand and the low natural regeneration of this species justify the need for an intensive plantation with an improved material. The current strategies of improvement of trees put the accent on the reproduction and the cloning of these trees in the ideal place. The techniques of the Micro distribution were used to surmount the problems related to the degradation of this forest heritage. The studies focus on the implementation of reliable protocols for the somatic embryogenesis [4], [5]. The application of this technique of regeneration can present several advantages among which we quote:

- The mass multiplication of the genotypes of trees elites of oaks-cork;
- A high rate of multiplication;
- The large-scale production;
- The genetic transformation, the cryopreservation of embryos;
- The possibility of direct transfer in the field or in greenhouse thanks to artificial seeds.

By consequence, the combination of this technology can be very useful in a program of improvement of oak [6], [5]. The Production of embryos by secondary or recurring embryogenesis is the stage giving to the somatic embryogenesis a multiplicative potential for the colonel mass distribution [7], [1]. However, little attention has been devoted to define the best conditions for the embryo proliferation stage, including embryo development. Despite the apparent feasibility of multiplying oak embryo genic lines by repetitive embryogenesis, the generation of secondary somatic embryogenesis developed up to the torpedo/cotyledonary stage (bipolar

structures) is not efficient in many oak embryogenic lines [8].

The secondary embryos resulting from superficial individual cells by multicellular budding, usually in the hypocotyle of the embryo mother [9], [10]. The Origin of embryo is particularly relevant for the genetic uniformity of the regenerated plants. As a multicellular origin can lead to the formation of genetically variable organisms, a unicellular origin is the way wished for the practical applications of cloning of embryos such as the genetic transformation [11].

The multiplication of embryogenic lines by secondary embryogenesis was realized most of the time by using culture medium containing the cytokinines as BAP, the auxines as ANA or AIB for Q. suber [12], [13], [14] or the 2, 4-D for Q. robur [15], [16] (chalupa 1993, Ostrolucka M.G 1996). More rarely the BAP alone or in combination with AG3 was used for Q. petraea (Jorgensen et al., 1993), Q. robur [15], [16] and Q. acutissima [17]. Concerning Zeatine single or in combination with the ANA, it was successfully also used in Q. robur [18].

The importance of a carbon source in the plant tissues culture is recognized for a long time. Carbohydrates are among five categories of substances which are necessary for the growth and for the organized development [19]. The source of carbohydrates plays an important role as source of energy and for osmolarity preservation [20], [8]. In the cultivated plant tissues, a continuous supply in carbohydrates from the environment is necessary, because the photosynthetic activity of in vitro tissues is reduced by the influence of the low luminous intensity, the limited gaseous exchanges and the high relative humidity [21]. In the culture of tissue, the apical and root morphogenesis is strongly dependent on the contribution in carbohydrates and it is essential to identify the specific needs during the various phases of the micro distribution [22]. The replacement of the medium carbon source of culture by osmotic activities solutions showed that sugars act as source of carbon and as osmotic regulators [23].

The sugar compounds normally present in perforated plant exudates have been positively related to the appropriate carbon source for use in the in vitro culture medium [24]. Sucrose is the most common sugar in the phloem Angiosperms species [25]. It has been used as a carbon and energy source, and at high concentrations it enhances somatic embryo maturation by causing osmotic stress [26]. Generally, the accumulation of storage products turns translucent embryos into white-opaque embryos. This change has been used as a maturity indicator in several species, including cork oak [27], olive [28] and avocado [29].

Although there are several published works concerning the adoption of the primary somatic embryogenesis technique applied to several species and specially to the Moroccan cork oak, However, data and information concerning the process of secondary somatic embryogenesis still rare or absents in the case of Moroccan cork oak.

The present work consists to testing the technique of secondary somatic embryogenesis on the Moroccan cork oak. The Maamora forest was chosen as example of application for its importance at Mediterranean scale. The experiments conducted here aim to evaluate the role of carbon sources on the secondary somatic embryogenesis process applied to the cork oak regeneration.

II. Materials and Methods

Embryogenic Lines used in these experiences were thrown between 2016 and 2017 from explants of sheets

resulting from adventitious shoots developed on branches segments. The starting explants have been isolated. They represent mature somatic embryos of (8-10 mm) of length at the cotyledonary stage. They were taken from dicotyledonary embryogenic cultures, obtained from leaf cuttings from an elite tree located in the region of Maamora (Morocco: GPS: N: 34°03'029, w: 006°38'207). The somatic embryos are kept during 1 year by recurrent embryogenesis on a series of subculture in a medium without growth regulators according to the protocol described in references [30], [6].

The pH was fixed on 5, 8 before autoclaving in 120 ° C at the atmospheric pressure during 20 min. The primary embryos were placed in the proliferation medium formed by the macronutrients of SH [31].

Microelements and vitamin MS have been added by 0, 7% of Vitro agar and 100mg / l of myoinositol. The tests are made in sterile Petri dishes (90 mm of diameter) filled with 20 ml of culture medium and sealed with the Parafilm®. The incubation took place in 25 ± 2°C in the darkness. The tested of carbon sources are: Sucrose, Glucose, Fructose, Sorbitol and Mannitol. The concentrations used are ranged between 10 and 40 g/l for all sugars. Sucrose was tested alone or associated to the sorbitol. With the exception of fructose, all others sugars have been added to the medium before autoclaving.

For this study, 30 explants of somatic embryos were cultivated for every test in each of the realized treatments (the control medium and five types of sugars). All the somatic embryos were distributed in a homogeneous way between treatments. The experience was repeated three times, thus a total of 1890 embryos were used.

Statistical Analysis

After 8 weeks of culture, we proceeded to the enumeration of explants showing the secondary somatic embryogenesis (ESII), the viable clusters (CL) and the somatic embryos (SE) on cluster per explants (primary somatic embryo). Thereafter, the different results are analyzed using the statistical analysis SPSS 17.0 software [32]. A unidirectional analysis of variance (ANOVA) is realized. Multiple comparisons were made by using the test post-hoc of Duncan ($\alpha = 0, 05$).

III. Results and Discussions

The obtained results are presented in the Table (1). Pictures presented in the figures 1-5 show a qualitative comparison concerning the influence of various sugars on the morphology of embryos formed after 8 weeks of culture.

Although the glucose at the concentration of 30 g/l appears the most favorable for the secondary somatic embryogenesis induction. However, for others concentrations, the difference between the results obtained by three sugars (Glucose, Sucrose and Fructose) are partially low. The mannitol and the sorbitol are unfavorable for the secondary somatic embryogenesis induction and also for the generation of clusters and embryos formed on clusters. They showed an inhibition effect against the secondary somatic embryogenesis. This tendency can be observed in a clear way when we make the comparison with the results obtained using the control medium. However we can note a low response in the case of sorbitol at the concentration of 20 g/l (0, 03 ± 0, 03) regarding the secondary embryos formation, but this result remains without any signification.

Table I : Effect of the five sugars tested on the multiplication of secondary somatic embryos of Quercus suber after 8 weeks of culture in the darkness.

Concentration (g/l)		Secondary Embryos	Clusters	Embryos formed on clusters
0 (control medium)		0,13 ± 0,79 f	0,00 ± 0,00 cd	0,00 ± 0,00 de
Sucrose	10	1,53 ± 0,33 e	0,13 ± 0,63 abc	0,3 ± 0,14 cde
	20	2,77 ± 0,51 bcd	0,33 ± 0,13 a	0,63 ± 0,24 ab
	30	3,23 ± 0,56 abc	0,57 ± 0,15 a	1,3 ± 0,34 ab
	40	3,66 ± 0,44 ab	0,37 ± 0,09 abc	0,97 ± 0,28 bc
Glucose	10	1,87 ± 0,27 e	0,47 ± 0,28 ab	0,83 ± 0,2 bcd
	20	2,4 ± 0,29 cde	0,66 ± 0,12 abc	0,67 ± 0,26 cd
	30	4,23 ± 0,67 a	0,60 ± 0,12 a	1,63 ± 0,47 a
	40	2,3 ± 0,34 cde	0,37 ± 0,09 abc	0,6 ± 0,18 cde
Fructose	10	2,07 ± 0,37 de	0,33 ± 0,09 abc	0,47 ± 0,14 cde
	20	2,3 ± 0,40 cde	0,43 ± 0,10 ab	0,7 ± 0,2 bcd
	30	3,6 ± 0,56 ab	0,4 ± 0,12 ab	0,83 ± 0,27 bcd
	40	1,5 ± 0,32 e	0,27 ± 0,08 bcd	0,47 ± 0,16 cde
Mannitol	10	0,00 ± 0,00 f	0,00 ± 0,00 d	0,00 ± 0,00 e
	20	0,00 ± 0,00 f	0,00 ± 0,00 d	0,00 ± 0,00 e
	30	0,00 ± 0,00 f	0,00 ± 0,00 d	0,00 ± 0,00 e
	40	0,00 ± 0,00 f	0,00 ± 0,00 d	0,00 ± 0,00 e
Sorbitol	10	0,00 ± 0,00 f	0,00 ± 0,00 d	0,00 ± 0,00 e
	20	0,03 ± 0,03 f	0,00 ± 0,00 d	0,00 ± 0,00 e
	30	0,00 ± 0,00 f	0,00 ± 0,00 d	0,00 ± 0,00 e
	40	0,00 ± 0,00 f	0,00 ± 0,00 d	0,00 ± 0,00 e

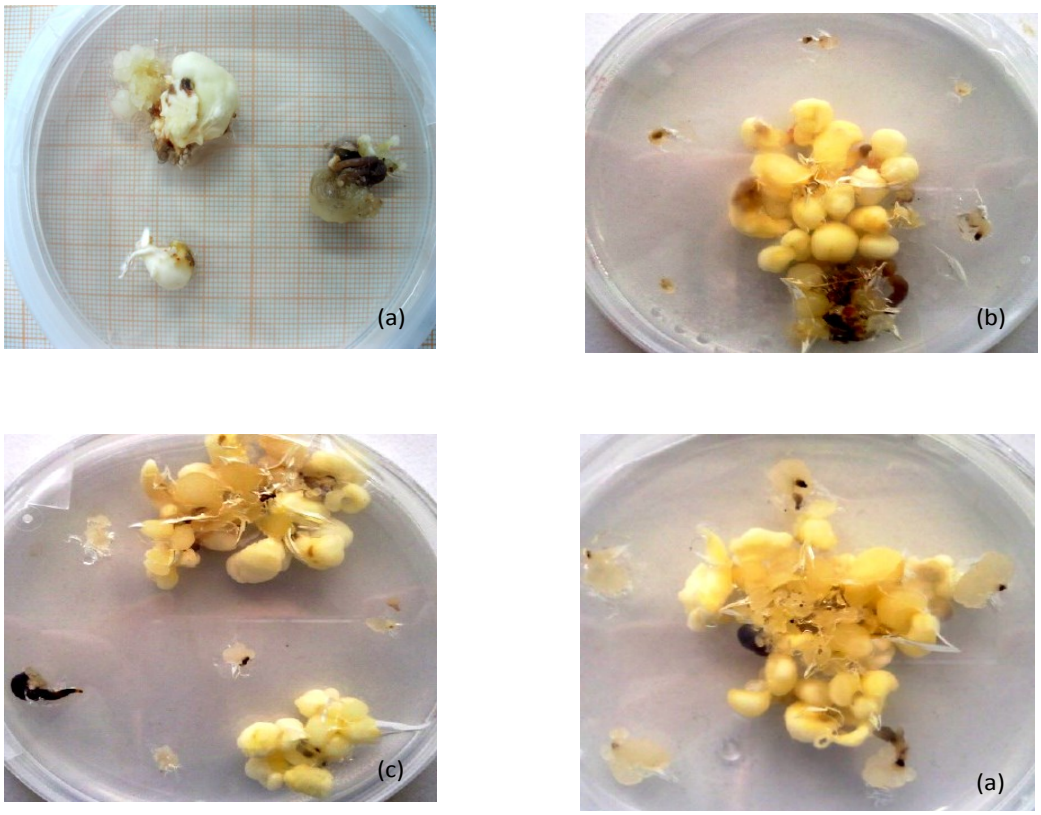

Fig. 1. Aspect of secondary somatic embryos: (a) control medium. (b) and (c) treatment with glucose. (d) treatment with sucrose. The photos were taken after 8 weeks of culture.

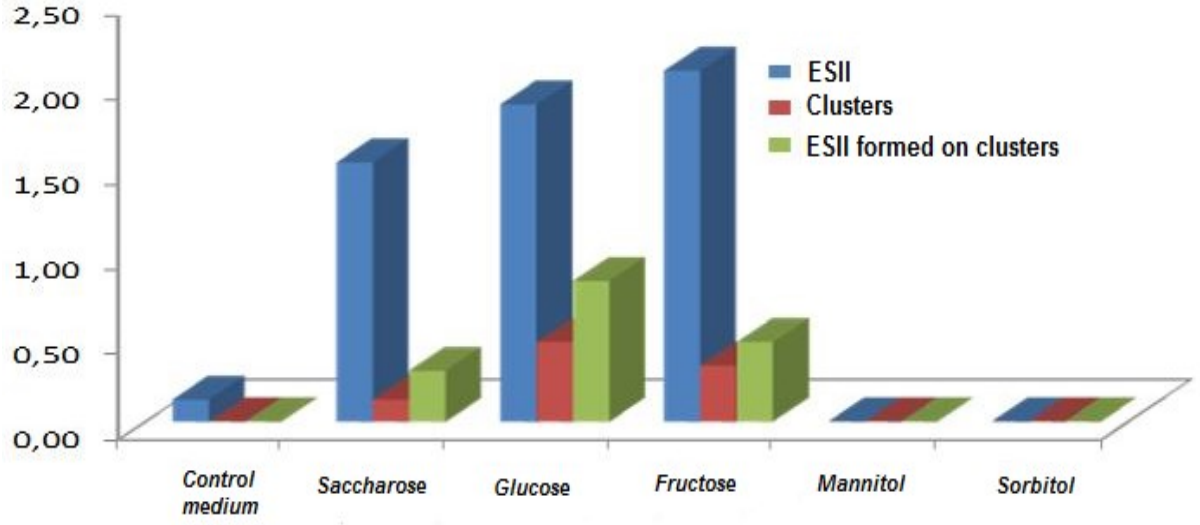

Fig. 2. Effect of five sources of carbon in 10 g/l on the multiplication of the secondary embryos, the clusters and the embryos formed on clusters after eight weeks of culture.

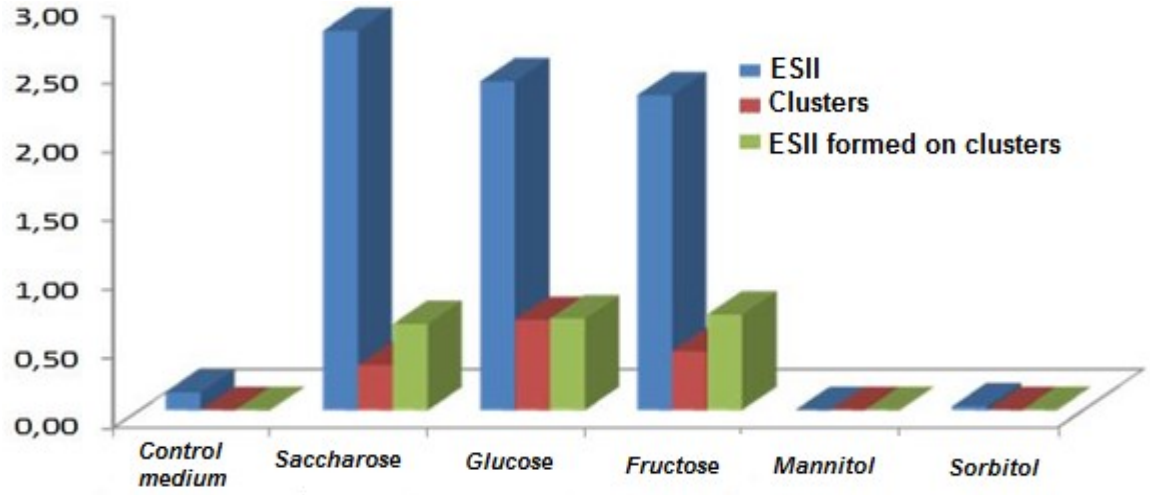

Fig. 3. Effect of five sources of carbon in 20 g/l on the multiplication of the secondary embryos, the clusters and the embryos formed on clusters after eight weeks of culture.

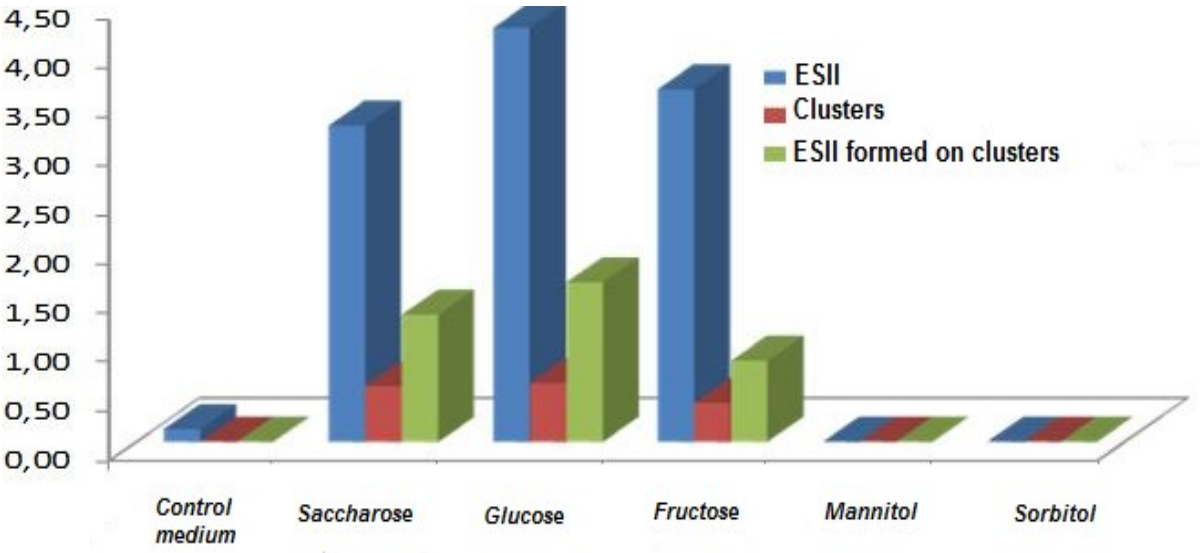

Fig. 4. Effect of five sources of carbon in 30 g/l on the multiplication of the secondary embryos, the clusters and the embryos formed on clusters after eight weeks of culture.

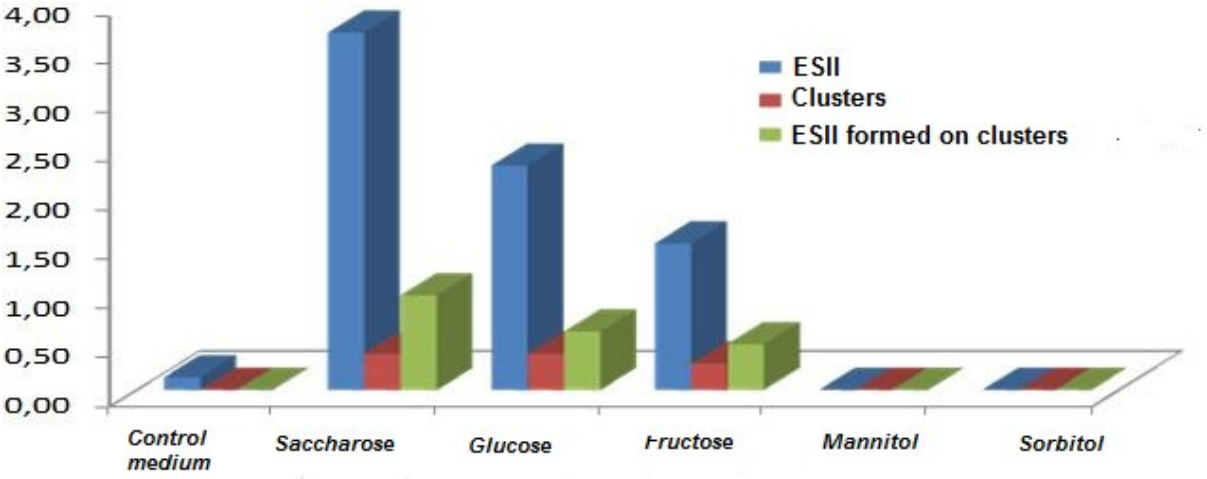

Fig. 5. Effect of the five carbon sources at 40 g / l on the multiplication of secondary embryos, clusters and embryos formed on clusters after 8 weeks of culture.

Table II: Effect of sucrose and sorbitol combination on the induction of secondary somatic embryogenesis of Quercus suber after 8 weeks of culture.

	Concentration (g/l)	Average Number of ESII	Average Number of clusters	Average Number of ESII formed on cluster
Control medium	-	0,11 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
Sucrose + Sorbitol	30 Sacc + 0 Sor	3,27 ± 0,54	0,60 ± 0,13	1,28 ± 0,35
	30 Sacc + 10 Sor	0,37 ± 0,20	0,03 ± 0,03	0,07 ± 0,07
	30 Sacc + 20 Sor	1,43 ± 0,41	0,33 ± 0,11	0,63 ± 0,21
	30 Sacc + 30 Sor	0,97 ±0,27	0,07 ± 0,07	0,06 ± 0,06

To confirm the inhibitor effect of the sorbitol, four different concentrations of sorbitol combined with the sucrose at concentration 30 g/l are tested. The results are represented in Table II.

This combination shows no significant results and remains widely lower than the response of the sucrose alone. The obtained result confirms those obtained previously. However, compared with the control medium, the averages obtained during this test are strongly due to the influence of sucrose and not to the sorbitol.

For the glucose and the fructose, the optimal concentration for the secondary somatic embryos induction is around 30 g/l. For the glucose and fructose the respective averages obtained for the SEII are 4,23 ± 0,67 and 3,6 ± 0,56 respectively.

For the sucrose, the maximum induction is recorded at 40 g / l. To verify if this concentration represents the optimum, a second series of tests was conducted covering concentrations ranged between 10 and 90 g/l. According to the obtained results (Figure 6), we note that the optimum for the SEII formation in the case of sucrose is around of 40 g/l. the average recorded for the secondary embryos is around 3,66 ± 0,44. The response in terms of number of clusters formed remains slightly insensible to the increase of the sucrose concentration (Figure 6). On the other hand, these results also confirm the results obtained in the previous experiments.

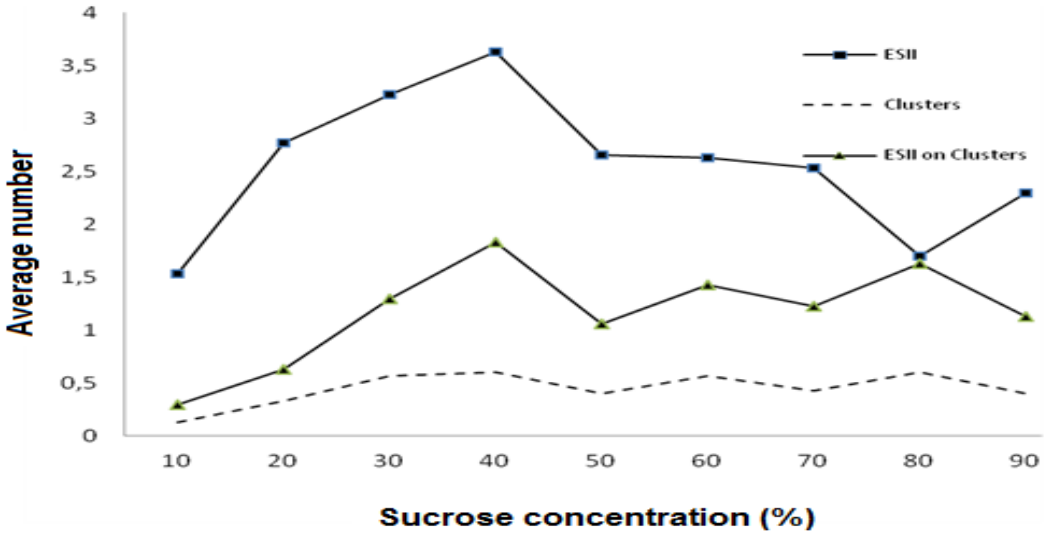

Fig. 6. Effect of sucrose on secondary somatic embryogenesis of Quercus suber after 8 weeks of culture in the darkness.

IV. Discussion

The main aim of this work was to investigate how the Carbone source can influence on the secondary somatic embryogenesis for Moroccan cork oak. Several studies showed that the chemical nature of sugars added to the circles of culture play a key role in the process of the morphogenic reactions. Among the various sources of carbon occurring in the differentiation and in the osmotic adjustment, sucrose stays the carbon source the most collectively used in vitro culture. Several studies [33], [34], [35] indicated that through his interaction with the growth regulators, the contents in sucrose of the culture medium affect the growth of the callus and afterward, the somatic embryogenesis.

In our case, sucrose showed favorable for the development of the secondary embryos, the clusters and even the embryos formed on these clusters. Nevertheless, we also notice that the increase of the concentration in sucrose in the culture medium environment until 90 g/l from 40 g/l is unfavorable for the induction of secondary somatic embryogenesis. However, the concentration of 40 g/l remains optimal for the development of secondary somatic embryogenesis. These results appear in agreement with those obtained by Gill et al [36] where the sucrose concentration higher than 50 g/l makes decrease or prevent the formation of somatic embryos of the geranium. Mauri et al [37] and Sanchez et al [9] affirm that the high concentration in sucrose (higher than 70 g/l) reduces significantly the rate of secondary embryogenesis and also the (germination). Besides, for other species, the induction and the development of the somatic embryos require higher concentration in sucrose such the case of the asparagus (50 g/l) [38] and the chrysanthemum (120-180 g/l) [39].

On the other hand, the obtained results show that the glucose turns out even more significant and distinguished compared to other sugars in the process of SEII (Figure 2). This result has also noticed by Romano et al [22] for Quercus suber L. As example, for Fagaceaes, it is demonstrated that the glucose at concentration 30g / l compared to sucrose or fructose gives better results from quantitative and qualitative point of view for the apex and cork oak [40], [22]. Also, other species show the advantage of the glucose compared to sucrose in the in vitro multiplication, as Castanea [41], Alnus glutinosa [24], Corylus avellana [42], Alnus cremastogine [43] and Prunus mume [44].

V. CONCLUSION

In the present study, we studied the effect of carbon sources on the induction of the secondary somatic embryogenesis of the Moroccan cork oak (Quercus suber L.) stemming from the forest of maamora. 5 types of sugars are tested through recognized protocols.

Although the sucrose was the carbohydrate of choice in the great majority of the works cited in the literature concerning the in vitro induction and the development of shoots of the woody species, it is demonstrated that this carbon source cannot always considered as the most effective in these purposes; through this study we demonstrated that the glucose is more effective for the secondary somatic embryogenesis process in the case of moroccan Quercus suber L.

The use of somatic mature embryos as primary explants allowed an effective induction of the ESII. However, the necessity of studying other parameters influencing this phenomenon of secondary embryogenesis stays among our major objectives to improve the in vitro regeneration of Moroccan cork oak.

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