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Callogenesis induction of *Ilex aquifolium* L. (Aquifoliales Aquifoliaceae)

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ABSTRACT English Holly (*Ilex aquifolium* L.) belongs to the family Aquifoliaceae and is one of the native and rare species in Algeria which has many benefits. The natural cultivation of this important ecological species presents many problems and takes a long time because of the various factors that increase the risk of extinction. In this research callogenesis was studied in order to induce better callus and to study the effect of Murashige and Skoog (MS), Woody Plant Medium (WPM) and various combinations and concentrations of growth regulators using young leaves as explants. Callus induction was successfully performed in WPM and MS culture media at high rates and with an earlier response on WPM medium. The maximum callus percentage (100%) was obtained on WPM medium supplemented with the combination of 6-benzylaminopurine (BAP) and naphthalene acetic acid (NAA) as well as on MS medium supplemented with BAP and 2.4-dichlorophenoxyacetic acid (2.4-D). The best mean callus surface area (151.77 mm^2) was obtained in WPM medium with (1 $mg.L^{-1}$of BAP+ 2 $mg.L^{-1}$ NAA). The addition of (2 $mg.L^{-1}$ BAP and 4 $mg.L^{-1}$ NAA) to the MS medium also produced a high mean callus surface area of 82.95 mm^2. The callus texture was compact and had three types of color: white, brown and greenish. The results of this study made it possible for the first time to develop an effective new alternative method for callus induction of *I. aquifolium*.

KEY WORDS Callogenesis; Combination; Growth regulators*; Ilex aquifolium;* Media.

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INTRODUCTION

Ilex L. Is the only living genus of the Aquifoliaceae family in tropical and temperate regions capable of surviving under most conditions (Savill, 2019). *Ilex aquifolium* L. is a slow-growing plant and one of the native and rare species in Algeria that should be protected, therefore encouraging its regeneration on appropriate sites (Yahi et al., 2008). Because of its ecological and ecosystemic importance, it has also generated commercial and economic activity (Guitián & Bermejo, 2006; Tsaktsira et al., 2018). The species has also been considered as a habitat of community interest and has an important role in biological and ecological diversity because it participates in a variety of forest communities because of its relationships with geopedological, climatic and anthropogenic characteristics. Its capacity to produce vigorous rejections of leaf masses makes it useful as a hedge species, so it represents a great scientific interest (Brunu, 2011). Extracts from different parts of the plant (leaves,

flowers, roots, etc.) have been used in traditional medicine to treat liver disease, stomach and intestinal cancer, rheumatoid arthritis, bronchitis and other inflammatory diseases (Nahar et al., 2005). These seeds are of particular importance in phytotherapy because they contain many derivatives with antioxidant activity (Nahar et al., 2006).

The natural and spontaneous regeneration of this species faces serious and difficult problems because of delayed germination and subsequent slow growth of new seedlings and late fructification (Minoglou & Panetsos, 1998). In effect, holly seeds exhibit a profound dormancy (quiescence) caused in part by the hard endocarp which inhibits development and in part by the immaturity of the embryo (Bonner & Karrfalt, 2008). Nevertheless, it experiences dormant periods of one year or longer and recent studies suggest a short persistence strategy of the species (Arrieta & Suarez, 2004).

Like most woody species, vegetative propagation, micropropagation and embryos culture techniques of *I. aquifolium* have been applied (Majada et al., 2000; Tsaktsira et al., 2018). However, even with the efforts made they are still insufficient, slow and at a low rate because of the material contamination during the culture process and low multiplication rates. Callogenesis is an important tool in basic and applied studies (it offers a large potential for use in pharmacology and pharmacy); so it has created new opportunities in commercial applications, agriculture and horticulture (Thorpe, 2012; Efferth, 2018). Several successful approaches have been made to develop callus induction protocols in other *Ilex* species (Dang et al., 2011; Stachevski et al., 2013).

The aim of the study was to develop an effective method and protocol for the induction of callus from young leaf explants. Therefore, this work aimed to evaluate the effect of various culture media and growth regulators combinations on callus induction in this species in order to determine the optimal conditions for the expression of maximal and reproducible callogenesis and the characterization of callus types obtained.

MATERIAL AND METHODS

To initiate callus, leaf explants of *I. aquifolium* were excised from seedling of six month old obtained by embryo germination under aseptic conditions in *in vitro* laboratory at National Research Center of Biotechnology of Constantine, Algeria.

To assess the effects of the media compositions and plant growth regulators (PGRs) on callus induction and proliferation two media were tested: MS (Murashinge & Skoog, 1956) and WPM (Lloyd & McCown, 1981) were each supplemented with four combinations of 6-benzyloaminopurine (BAP), 2.4-dichlorophenoxyacetic acid (2.4-D), Naphthalene Acetic Acid, 3% sucrose as a carbon source and 0.7% agar. The pH of the media was adjusted to 5.8 before autoclaving at 121 °C for 20 min.

The leaves were cut into small peaces and then cultured in 10 cm Petri dishes containing fresh media with combinations for callus induction (Table 1). Three explants per Petri dish were used for each treatment and all treatments were replicated six times.

Subculturing of explants was carried out in petri dishes at 4-week intervals for each combination and concentration of plant growth regulators. Cultures were incubated at (25 ± 2) °C in dark and subcultured within 30 days. To produce many more calli, good quality calli were transferred to fresh media based on callus number, induction rate and color. The experiment was replicated five times for 30–45 days according to callus development. Data were analyzed for the frequency of callus formation and proliferation. The parameters observed were callus formation time, color and callus textures, callus percentage and callus surface. The measurement of the surface was carried out on a millimeter ruler fixed on a sterile petri dish, measuring the diameters: D1 the widest and D2 the perpendicular. The surface is determined by the formula (Dale & Deambrogio, 1979):

$$\text{Calli area } (mm^2) = \frac{(D1 \times D2)}{2} \times \pi$$

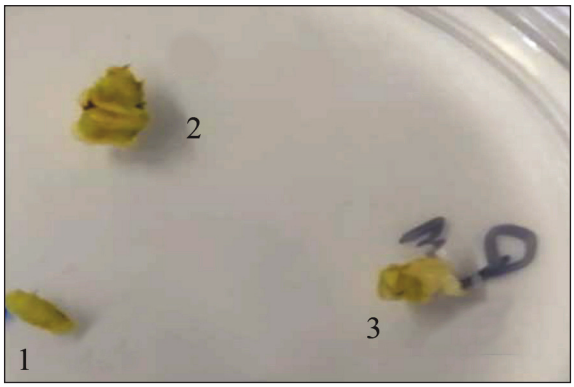
Data analysis

Data from callus induction and surface were analyzed using a one way of variance (ANOVA) in a multiple comparison based on a completely randomized design. P-values ≤ 0.05 were considered statistically significant. The Tukey test was applied to determine exactly which treatments were different ($p \leq 0.05$). All statistical studies were performed using SPSS Version 20–32 bit (IBM, USA).

RESULTS

In vitro, callus induction depended on both medium and PGRs combination. Analysis of variance showed that among each treatment (PGRs and medium) (Table 1). Callus induction was observed on the cut surfaces of leaves within one to two weeks and was reflected in the margins of the leaves and in the injuries given to the explants. The first step is the swelling of the explants, followed by cell division and significant anarchic proliferation of its cells completely covering the explants which will form a callus (Figs. 1–3).

The explants of *I. aquifolium* were able to grow on both WPM and MS media supplemented with different concentrations of growth regulators and began to appear after 12 days. Results in table 1 show that the primary callogenesis has a very diverse variation. The best initiation of callus was in WPM medium supplemented with 1 mg.L^{-1} BAP and 2 mg.L^{-1} NAA (Table1). The highest callus surface average was obtained when on WPM medium supplemented with 1 mg.L^{-1}BAP and 2 mg.L^{-1} NAA followed by 1 mg.L^{-1} BAP and 0.5 mg.L^{-1} 2.4 D. However, the double concentration of the same combination strongly reduced the surface average. Statistical analysis of the data reveals a significant difference between the two media with the identification of two types of homogeneous groups (a and b).



Figures 1–3. Callus induction. Fig. 1: callus formed at one end of explant. Fig. 2: grows on parts of explant surface. Fig. 3: grows on whole surface of explant.

The effect of several combinations of growth regulators tested to determine which ones achieve the highest percentage of callogenesis. All the combinations used induced the reactivity of the cultured explants. However, there were differences in response time and callogenesis rate after two months of culture (Table 2).

Indeed, on the WPM medium completed with (1 mg.L^{-1} BAP, 2 mg.L^{-1}NAA) and (1 mg.L^{-1} BAP, 0.5 mg.L^{-1} 2.4-D) were the fastest and first to show callogenesis. The results of Table2 show that maximum (100%) callus induction rate were recorded on treatments WPM + (1 mg.L^{-1} BAP + 2 mg.L^{-1} NAA) and WPM + (2 mg.L^{-1} BAP +4 mg.L^{-1}

Combinations				**Callus reactivity/medium**		**Callus surface average/medium**	
Treatment	**BAP**	**2.4- D**	**NAA**	**WPM**	**MS**	**WPM**	**MS**
1	1	0.5	-	++	++	100.04±18.21^{b}	31.31±23.14^{c}
2	2	1	-	+	++	33.06±35.54^{c}	30.79±32.05^{c}
3	1	-	2	+++	+++	151.77±65.38^{a}	40.38±22.19^{b}
4	2	-	4	++	+++	19.28±27.57^{d}	82.95±53.14^{a}

Table 1. Effect of growth regulators and different media on explant responses and callus average surface of *Ilex aquifolium.* Means followed by the same letter are not significantly different ($p \leq 0.05$). +: explant swells or callus formed at one end of explant; + +: callus grows on parts of explant surface; + + +: callus grows on the whole surface of explant.

NAA), and a high rate of (94.44%) was recorded on treatments MS supplemented with (1 mg.L^{-1} BAP, 0.5 mg.L^{-1} 2.4-D) and (2 mg.L^{-1} BAP, 1 mg.L^{-1} 2.4-D). Statistical analysis reveals a non-significant difference between the different treatments applied to the WPM medium (Table 2). While on MS, the explants in the presence of all treatments had the same callus initiation duration and the percentage of callus differed significantly among the treatments.

The results of ANOVA showed that, among each treatment, the best callus type and callus induction rate (100%) was recorded on treatments MS + (1 mg.L^{-1} BAP + 0.5 mg.L^{-1} 2.4-D) and MS + (2 mg.L^{-1} BAP +1 mg.L^{-1} 2.4-D) (Table 2).

Compact white callus was obtained with all treatments except with the high concentration of BAP and NAA (2 mg.L^{-1}+ 4 mg.L^{-1} respectively) that gave brownish callus on both mediums, whereas greenish callus was observed only on MS medium with 1 mg.L^{-1} BAP and 0.5 mg.L^{-1} 2.4-D (Table 2 and Figs. 4-6).

DISCUSSION

Callus reactivity varies according to several factors such as the type of medium and the combinations of growth regulators tested. Astuti et al. (2020) have shown that growth regulators are very important in accelerating callus formation and growth. According to Galáz-Ávalos et al. (2012), the behaviour of the cells is different at each stage of callogenesis. The difference in the primary reactivity of the callus in the treatments can also be explained by the fact that the size of the sheets that were used as explants was not completely uniform; this may have an influence on the amount of call tissue (Suwanseree et al., 2019).

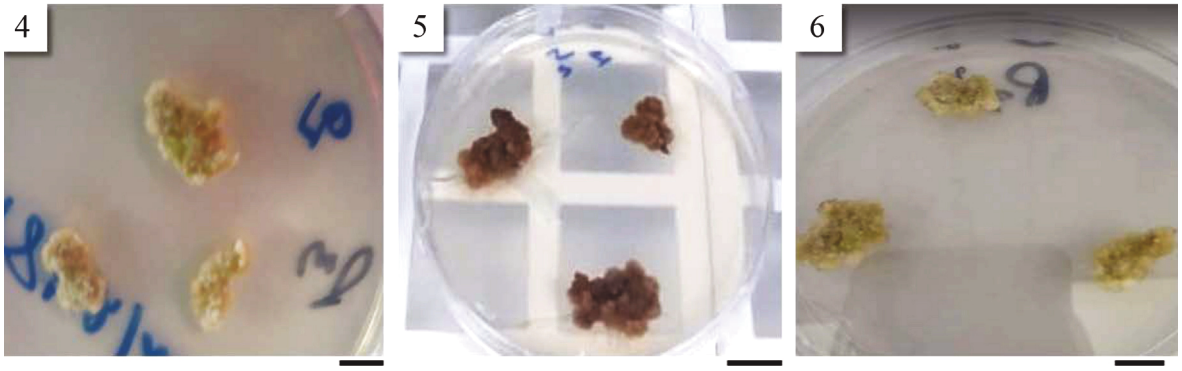
The callus surfaces revealed significant differences between the culture media. According to the results, the WPM medium has a higher average total surface area than the MS medium. This difference can be explained by the effect of the variation of the mineral elements that constitute them and all the interactions between these elements. Some of them stimulate *in vitro* development processes, while others have a lesser influence on development (Telahigue & Toumi, 2017).

According to Munazir et al. (2010) and Astuti et al. (2020), callus can be induced but there is not a sufficient proliferation that leads to a decrease in development, due to the fact that callus have not yet reached the stage of differentiation.

Callus surface diversity may be related by the interaction between endogenous phytohormones of the explants and growth regulators absorbed from the culture medium, which will influence the callus development (Asnawati et al., 2002). The sizes of the different callus obtained in each medium completed by different treatments of growth regulators can be caused by the different capacity of absorp-

Combinations (mg.L^{-1})				Percentage of callus induction/medium		Type of induced callus/medium		Type of callus	
Treatment	**BAP**	**2.4-D**	**NAA**	**WPM**	**MS**	**WPM**	**MS**	**WPM**	**MS**
1	1	0.5	-	94.44^{a}	100^{a}	W	Gr	C	C
2	2	1	-	94.44^{a}	100^{a}	W	W	C	C
3	1	-	2	100^{a}	94.44^{a}	W	W	C	C
4	2	-	4	100^{a}	83.33^{b}	B	B	C	C

Table 2. Effect of growth regulators and different media on callus formation of *Ilex aquifolium.* Means followed by the same letter are not significantly different ($p \leq 0.05$). B: brown; W: white; Gr: greenish; C: Compact structure.



Figures 4–6. Callus induction of *Ilex aquifolium*. Fig. 4: white callus.
Fig. 5: brown callus. Fig. 6: green callus. Scale bar 1 cm.

tion of water and nutrients, as well as the ability to hold the process of diffusion, osmosis and turgidity pressure of the cells. These factors are also provided by Maulida et al. (2019).

The results concerning the rate of callogenesis on both media can be explained by the reason that each species has different nutrients at different concentrations for *in vitro* culture. The WPM medium has a more diluted mineral formulation (lower ionic strength) compared to the MS medium, one of the main differences between them concerns their salt concentrations, especially nitrogen and potassium, which are lower in the WPM (Zaniolo & Zanette, 2001). Similar results were obtained by Saini et al. (2014), where calls induction of *Mesua ferrea* L. (Malpighiales Calophyllaceae) was higher on both media WPM and MS, but the induction duration was short on WPM media. The superiority of the WPM medium in producing an earlier callogenesis response has also been reported by Behbahani et al. (2011), Osman et al. (2016), El Bouzdoudi et al. (2017). It is also mentioned that among the compositions of WPM medium, another nitrogen source which is CaNO3, while in MS medium, nitrogen is provided by KNO3 which suggests that the combination $NH4^+$ $NO3^-$ and CaNO3 is more efficiently promoting callus induction. However, in the experiment of Stachevski et al. (2013), the best callus growth rates of *I. paraguariensis* A. St-Hill. were obtained in the MS Medium.

All growth regulators combinations tested (auxins: 2,4D or NAA) combined with a cytokinins (BAP) at various concentrations produced callus in different ways, the highest rate of callus induction in WPM and MS media can be caused by the endogenous hormone present in the explants responsible to determine the callus induction capacity and their interaction with various exogenous hormones (Kiranmai et al., 2015; Yulianti, 2015). Callogenesis abilities depend on many parameters, and traditional methods of plant regeneration from callus involve the manipulations of the auxins to cytokinins balance (Thomas & Maseena, 2006). In our study and on the WPM medium the low concentrations of auxins by contribution to cytokinins had a better effect on callus inductions in contrast to those of the MS medium. The nature, concentration, and combination of growth regulators further affect callus induction (Grunennvaldt, 2008). WPM added with low concentrations of 2.4-D and NAA were the first to express callogenesis and the addition of BAP to the culture media significantly improved the induction of callogenesis, our results are similar to those of Telahigue and Toumi (2017). The synergistic effect of BAP with NAA or BAP with 2.4-D to promote callus initiation and formation is confirmed by many authors (Ndoumou & Tchinda, 2008; Chaâbani et al., 2015). The NAA/BAP combination with WPM produces more callogenesis than the 2,4D/BAP combination. The same results were obtained by El Bouzdoudi et al. (2017). However on MS medium, the 2.4-D/BAP combination produced the highest rate of call induction, these results are also in agreement with those of Dang et al. (2011) on *I. khasiana* Purakaystha.

The nutrients and components of the media have an effect on the morphogenetic responses of the cultivated species, because there are great differences in the content of macronutrients and micronutrients in the different basal culture media (Saad & Elshahed, 2012). According to Fitriana et al. (2019), the compact texture of the calli is generally considered good because it accumulates more secondary metabolites. Furthermore, compact calli are considered the best kind of in vitro selection and plants regeneration (Leupin, 2000). Compact calli were also obtained from the leaves of *I. paraguariensis* (Stachevski et al., 2013). Callus color indicates the presence of chlorophyll in the tissue, where the callus is more green, the chlorophyll content is higher, which could be caused by a cytokinin effect. The white callus in an embryogenic tissue indicates that the callus state is relatively good (Ariati, 2012). According to our results, the brown calli may be caused by the high concentration of NAA. Similar results were reported by Al-Mayahi et al. (2018), where the calli became brown in the medium containing high auxin content.

CONCLUSIONS

To date there has been no report of *I. aquifolium* L callus induction. Callus induction and proliferation seem to be controlled more by the concentrations of auxins (2,4-D and NAA) than by cytokinins (BAP) regardless of the culture medium. Nevertheless, among the various concentrations and combinations of BAP and 2,4-D, WPM supplemented with BAP ($1mg.L^{-1}$) and NAA ($2mg.L^{-1}$) was found to be the best to induce early callus of *I. aquifolium*. The highest surface callus was obtained on Ms supplemented with high concentration of growth regulators combination. Three kinds of calluses were obtained; the majority of them were compact and white, brown and greenish in color.

The results of this study provide evidence that the in vitro culture protocol which we developed can be a practical means for micropropagating and preserving this endangered species through indirect and direct organogenesis and to transfer in vitro the plantlets to the field. Further studies on this topic are desirable.

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