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Research Article

Induction of organogenesis and callogenesis in *Limbarda crithmoïdes* L. (Asteraceae) explants cultured on MS media supplemented with various concentrations of Na^+ and K^+

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Abstract

The present work describes an efficient micropropagation and callus induction protocol of *Limbarda crithmoïdes* L. (Asteraceae), a halophyte species with medicinal and horticultural interests. The objective was to identify the culture media that support the best organogenesis and callogenic expressions of four types of aerial explants; nodal and internodal segments and leaf portions, by varying the mineral composition of twenty culture media by adding increasing concentrations of Na^+ (from 0-100 mM) and K^+ (from 0-50 mM). After two months of culture, parameters relating to the various expressions of organogenesis and callogenesis were measured. Results showed that the K^+ and Na^+ interactions affect the explants development. The combination of high concentrations of Na^+ (50, 100 mM) and K^+ (50 mM) ions allowed the most important regeneration of the axillary shoots (70.6-100%), root neoformation (82.6-96.1%) and callus induction (76.3-100%). Rooted plantlets with well-developed axillary shoots have been successfully acclimatized with a 93% of survival rate. The selected media would allow a large-scale multiplication of this medicinal species, without adding exogenous phytohormones, and could be used for the micropropagation of other threatened halophyte species or for the production of callus; rich in secondary metabolites.

Keywords: callogenesis; *Limbarda crithmoïdes* L.; organogenesis; potassium; sodium

Introduction

The Asteraceae (Compositae) was considered as the largest family of flowering plants including 25 000 species distributed in about 1500 to 1700 genera (Giberti, 2018). They are widely dispersed in all continents and occupied several types of habitats (Mandel *et al.*, 2019). *Inula* L. genus, also known as *Dittrichia* Greuter. or *Limbarda* Adans. (Le Floc'h *et al.*, 2010), belongs to the Asteroideae subfamily and the Inuleae tribe

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(Anderberg, 2011). It includes more than 100 species distributed in Africa, Asia and in the Mediterranean region (Seca *et al*., 2014). In Tunisia, it is represented by four species; *Dittrichia viscosa* (L.) Greuter subsp. *viscosa*, (syn. *Inula viscosa* (L.) Aiton), *Dittrichia graveolens* (L.) Greuter (syn. *I. graveolens* (L.) Desf.), *Inula montana* L. var. *calycina* and *Limbarda crithmoïdes* (L.) Dumort. subsp. *longifolia* (Arcang.) Greuter (syn. *Inula crithmoïdes* L.) (Le Floc'h *et al*., 2010). *Limbarda crithmoïdes*, well known as Golden samphire, is a succulent perennial coastal plant commonly found in the Mediterranean basin in salt water marsh areas (Zurayk and Baalbaki, 1996). It displays a fascinating capacity to tolerate high sodium concentrations in its aerial parts (Flowers and Colmer, 2008; Vicente and Boscaiu, 2020) allowing it to have salt, water, and metallic stress tolerance (Al Hassan *et al.*, 2016; El-Sherbeny *et al.,* 2021; Dridi *et al.*, 2022a; 2022b). *L. crithmoïdes* has long been collected for its medicinal properties and therapeutic uses. It was traditionally consumed especially in curing and preventing goiter, due to its high iodine shoot content and its roots were used in folk medicine as a tonic or diuretic (Philips, 1957). Moreover, it was a rich source of a wide range of bioactive compounds; flavonoids, phenolic compounds, tannins, alkaloids, essential oils, terpenoids, glycosides, and polysaccharides (Belloum *et al.,* 2013; Jallali *et al*., 2014; Jdey *et al.*, 2017; Roux *et al*., 2017; Das *et al.,* 2020; D'Agostino *et al*., 2021; Gharred *et al.*, 2022) which have shown interesting biological activities such as antioxidant, anti-inflammatory, antitumor, antidiabetic, antileishmanial, antimicrobial, antifungal, antibacterial and allelopathic (Omezzine *et al.*, 2011; Aboul Ela *et al.,* 2012; Jallali *et al*., 2018, Oliveira *et al*., 2018; Omezzine *et al.*, 2019; Adorisio *et al*., 2020; Mzoughi and Majdoub, 2021; Gharred *et al.*, 2022). It also has an agronomic potential in saline agriculture (Lima *et al*., 2021) for human consumption as an edible halophyte (Tardio *et al*., 2006). Young succulent leaves were eaten raw in salads or cooked as a leaf vegetable (Kunkel, 1984). This species was occasionally used as a pot herb (Facciola, 1990), and considered an ornamental and horticultural plant in the HALOPH Database of salt-tolerant plants of the world (Aronson, 1989). It has also good potential as a quality animal feed supplement in view of its high crude protein content (Zurayk and Baalbaki, 1996). Besides, Ghabriche *et al.* (2017) have studied its cadmium toxicity response and then its phytoremediation uses and Salem *et al.* (2019) highlighted its considerable lignocellulosic biomass which can be used in bioethanol and high-quality paper productions.

To the best of our knowledge, no previous research on the micropropagation of this species has been conducted, except this of Bucchini *et al*. (2013) in Italy, where authors investigated the phenolic content of its callus cultures grown on Murashige and Skoog (MS) medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D). On the other hand, there are no reports of mineral balance use for inducing root and shoot regeneration or callus induction in any *Inula* species. All the micropropagation studies of *Inula* species are made by adding growth substances in media culture. Besides, research works studying the effect of the mineral balance on *in vitro* organogenesis are very scarce. From which, we quote works of Harzallah-Skhiri (2003) and Stambouli *et al.* (2011; 2012) for the Tunisian native *Prosopis farcta* (Banks &Soland.) Macbride and that of Benrebiha *et al.* (1992) for *Atriplex halimus* L. Mineral balance is crucial and have an important impact on the plant *in vitro* development (Niedz and Evens, 2007; Aranda-Peres *et al.,* 2009; Efferth, 2019). The optimum mineral content of the culture medium varies significantly depending on the species or genotype for a successful micropropagation procedure (Cézar *et al*., 2015; Reed *et al*., 2016). Indeed, there is very little known of the relationships between mineral uptake and *in vitro* morphogenesis (Ramage and Williams, 2002). According to Poothong and Reed (2014), a few information was available on *in vitro* mineral nutrition, although it represents one of the main factors of the micropropagation of plants.

The present research was conducted in the objective to study the *in vitro* organogenesis and callogenesis expression of the halophyte *L. crithmoïdes* explants by increasing concentrations of sodium and potassium, in media culture. Establishment of the nutritional requirements of cultured explants and the optimization of the composition of the *in vitro* culture media, based on a defined mineral balance, without exogenous hormones

use, is therefore a challenge allowing further safeguard and conservation of this medicinal species and of other threatened halophytes.

Materials and Methods

Plant material

Aerial parts were randomly harvested in March 2021 during full flowering stage from *L. crithmoïdes* plants growing wild on coastal areas and maritime cliffs of Skanes (Latitude: 35°78' N, Longitude: 10°80' E), located at 3 km from Monastir; East of Tunisia. This species was identified by qualified botanist Pr. Fethia Harzallah-Skhiri and voucher specimens (*AsLc*,1-5, 21) were deposited at the Herbarium of the Laboratory of Bioresources: Integrative Biology and Valorization at High Institute of Biotechnology of Monastir, Tunisia.

Sterilization protocol

Leaves and stem cuttings (4 cm length with 4-5 nodes) were thoroughly washed under running tap water in order to remove dirt and dust, then soaked in Pril detergent solution (10%, v/v) (Henkel Group) for 15 min, rinsed 3 times with distilled water, and chemically sterilized under a laminar air flow hood. In fact, an optimum sterilization procedure, allowing the maintenance of cultures free from microbial contaminations, has been defined after several preliminary tests. So, plant material was dipped in ethanol (70%, v/v) for 1 min, then in sodium hypochlorite solution (5% and 10%, v/v), for 3 and 5 min, respectively), and finally sterilized with $HgCl_2$ (0.2%, w/v), for 5 min and rinsed 4-5 times with sterile distilled water.

Explants preparation

Sterilized tissues separated in nodal and internodal segments of approximately 10 mm length and in portions from distal and proximal leaf sides (10 mm^2) were aseptically dissected and incubated in a solution containing 300 mg/L ascorbic acid for 2 h in order to limit phenolic explant exudation. Under laminar air flow hood, the obtained micro cuttings are implanted vertically (stem explants) and horizontally (leaf explants) in 25×150 mm culture tubes. A total of 1 920 explants were used (24 repetitions per explant for each culture medium tested).

Culture media

The composition of twenty defined culture media was prepared from the full-strength Murashige and Skoog's basal medium (MS) (Murashige and Skoog, 1962). For each medium, we calculated the anions and cations contribution or supply in order to maintain the ionic balance while taking in account the concentrations of potassium and sodium contained in the MS medium. Potassium (K^+) and sodium ions (Na^+) molar concentration were reported in Table 1. The choice of the mineral cations' types, as well as their respective concentrations, were based on a soil test. In fact, a previous analysis of soil where *L. crithmoïdes* grows spontaneously shows that sodium and potassium exhibit the highest concentrations in minerals. In all the defined media, we added vitamins of MS, sucrose (30 g/L), EDTA-Fe (0.1 mM/L) and 8 g/L of agar (Bactoagar-Difco). The final pH was set to 5.8. Culture was incubated in a controlled growth chamber at 24±1 °C under a 16/8-h (light/dark cycle) photoperiod provided by cool white light fluorescent tubes 36 W Osram (intensity of 50 $\mu E\ m^{-2}\ s^{-1}$).

Table 1. Concentration (mM) of K^+ and Na^+ in the twenty-culture media defined

K^+ (mM)	Na^+ (mM)			
	0	**10**	**50**	**100**
0	M1	M2	M3	M4
1	M5	M6	M7	M8
5	M9	M10	M11	M12
10	M13	M14	M15	M16
50	M17	M18	M19	M20

M: medium

Measured parameters

Data were collected after two months of culture. The percentages of explants that initiated adventive roots (%exaR), shoots (%exS), or formed callus (%exCal), were recorded. The number of adventive roots per explant (Nb.aR/ex), the numbers of secondary roots per adventive root (Nb.sR/aR), of shoots per explant (NbS/ex), and of regenerated leaves per explant (Nb.le/ex) were noted. Moreover, adventive root length (cm) (aRL), shoot length (cm) (SL), leaf length (cm) (leL) and callus area (mm^2) (CalA) (Dale and Deambrogio, 1979), were measured and developed fresh callus (mg) (FwCal) was weighed.

$$CalA\ (mm^2) = (D1 \times D2/2) \times 3.14 \quad (1)$$

D1: length of the largest callus diameter; D2: length of the callus diameter perpendicular to D1.

Acclimatization

The root system of thirty *in vitro* regenerated plantlets from nodal explants collected after 8 weeks was washed carefully with sterile distilled water. Subsequently, these were transplanted to Jiffy-pots (6 cm diameter and 750 cm^3 volume) filled with a mixture of sterile peat commercial soil substrate (2/3) and fine sand (1/3), placed under a greenhouse at 23 °C day/18 °C night temperature and watered after every 2 days. The survival rate was calculated after 3 months of culture.

Statistical analyses

The data were analyzed using analysis of variance (ANOVA). The significance of the differences between means was determined at $p < 0.05$ with the post-hoc Tukey test. All the measured parameters obtained within each media culture were subjected to Principal Components (PCA) and Hierarchical Clusters (HCA) Analyses. Both methods made it possible to classify media into groups and to identify those favoring the most effective expression of organogenesis and/or callogenesis using XLSTAT 2016 (on windows 10) and the statistical R Studio (version 3.5.1) on Linux (Ubuntu) software's.

Results and Discussion

Effects of potassium and sodium ions on the caulogenesis

Shoot induction started within two weeks of culture only from nodal segments. The intensity of *in vitro* shoots direct induction on nodal explants varied significantly according the twenty tested media (Table 2).

PCA (Figure 1A) and HCA (Figure 1B) analyses classified the results into three groups of media. The group 1 formed by five media (M7, M8, M17, M19 and M20) which promote the best direct shoot regeneration. All controlled parameters were at the highest values for explants incubated in M17 medium containing 50 mM K^+ without Na^+. Moreover, an important caulogenesis responses are obtained on explants developed in media added with high Na^+ concentrations; 50 mM or 100 mM, with a supply of 1 mM (M7, M8) or 50 mM (M19, M20) of K^+ (Figures 3 E, G).

Explants grown in media from group 2 (M1, M2, M3, M4, M6, M13, M14, M15, M16 and M18), have lower values for all caulogenesis variables than the previous group. In fact, whatever the Na^+ concentration (1-100 mM), when the medium was free of K^+ (M1, M2, M3 and M4) or it contains 10 mM (M13, M14, M15 and M16), the values of the controlled parameters were weak. When the Na^+ is at 10 mM and K^+ at 50 mM (M18), or when Na^+ is at 10 mM and K^+ at 1 mM (M6), values of the controlled parameters are average or moderate. Besides the shoot rate regeneration, the other parameters follow the same pattern.

Media from group 3, containing potassium concentration of 5 mM with increasing concentrations of sodium (M9, M10, M11 and M12) or of 1 mM and free of sodium (M5) are not suitable for shoot regeneration from nodal segments. All the analysed parameters have very low values.

Table 2. Effect of different combinations of K^+ and Na^+ in M1-M20, on direct caulogenesis from nodal explants of *L. crithmoïdes*

Media	Nodal explants				
	%exS	NbS/ex	SL	Nb.le/ex	leL
M1	*20.1 ± 0.8 i	1.3 ± 1.45 i	6.2 ± 0.04 g	22.2 ± 0.3 l	3.1 ± 0.4 g
M2	20.6 ± 0.6 i	1.5 ± 0.2 h	5.2 ± 0.6 i	19.1 ± 0.2 m	4.1 ± 0.5 cd
M3	20.9 ± 0.9 i	0.9 ± 0.4 k	6.6 ± 0.2 f	35.6 ± 0.7 h	3.7 ± 0.9 ef
M4	20.2 ± 0.3 i	2.5 ± 0.4 g	6.7 ± 0.6 e	45.0 ± 0.4 f	3.0 ± 0.8 g
M5	0.5 ± 0.3 j	0.3 ± 0.1 n	0.6 ± 0.2 m	0.6 ± 0.6 o	0.2 ± 0.5 h
M6	44.2 ± 0.8 f	1.1 ± 0.7 j	7.0 ± 0.8 c	40.3 ± 1.4 g	3.1 ± 2.1 cd
M7	60.1 ± 0.4 d	2.9 ± 1.8 f	7.1 ± 0.6 d	58.2 ± 0.2 d	4.1 ± 0.3 cd
M8	61.2 ± 0.2 d	3.6 ± 0.9 d	7.3 ± 0.3 c	66.2 ± 0.8 c	4.2 ± 1.6 c
M9	0.6 ± 0.2 j	0.6 ± 0.l m	1.0 ± 0.3 k	0.7 ± 0.5 o	0.2 ± 0.2 h
M10	0.4 ± 0.0 j	0.5 ± 0.2 m	1.2 ± 0.5 j	0.3 ± 0.2 o	0.1 ± 0.2 h
M11	0.5 ± 0.1 j	0.3 ± 0.1 n	0.7 ±0.1 lm	0.1 ± 0.1 o	0.1 ± 0.5 h
M12	0.4 ± 0.4 j	0.1 ± 0.1 o	0.8 ± 0.4 kl	0.1 ± 0.9 o	0.1 ± 0.1 h
M13	30.3 ± 0.1 h	1.1 ± 0.6 j	5.2 ± 0.5 i	12.1 ± 0.7 n	3.6±1.8 f
M14	40.1 ± 0.2 g	0.9 ± 0.3 k	5.3 ± 1.8 h	25.5 ± 1.3 k	3.9 ± 0.1 e
M15	30.0 ± 1.3 h	1.5 ± 0.8 h	6.8 ± 0.5 e	32.2 ± 0.6 i	3.9 ± 0.8 e
M16	30.2 ± 0.6 h	1.5 ± 0.7 h	6.6 ± 0.6 ef	30.0 ± 0.8 j	3.9 ± 1.1 de
M17	100.0 ± 0.5 a	8.3 ± 1.2 a	10.1 ± 0.4 a	70.6 ± 1.8 a	5.1 ± 0.4 a
M18	53.1 ±1.2e	3.1 ± 0.4 e	7.3 ± 1.4 cd	50.8 ± 0.6 e	4.2 ± 0.3 g
M19	70.6 ± 0.2 c	3.9 ± 0.5 c	7.5 ± 0.6 c	66.5 ± 1.3c	4.3 ± 0.7 bc
M20	80.0 ± 0.4 b	4.2 ± 1.2 b	7.7 ± 0.8 b	68.9 ± 0.7 b	4.5 ± 0.6 b

***** Note: Data represents the mean of three replicates. The means ± the standard deviations followed by the same letter within the same column are not significantly different ($p > 0.05$) (Tukey's test). Culture duration was 2 months.
%exS; Percentage of explants that initiated shoots, NbS/ex; Number of shoots per explant, SL; Shoot length (cm), Nb.le/ex; Number of regenerated leaves per explant, leL; Leaf length (cm)

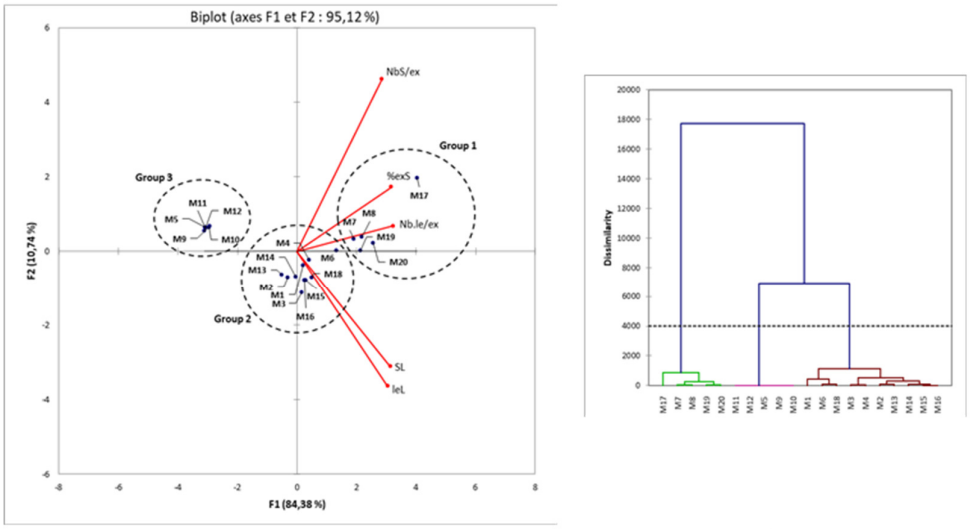

Figure 1. Distribution of the twenty media (1-20) containing varying concentration of K^+ and Na^+, in the plans defined by the axis 1 and axis 2 of the PCA (A) and HCA (B) analyses based on five caulogenesis variables (%exS, NbS/ex, SL, Nb.le/ex and leL) defined for nodal explants incubated in each of those media

The obtained results concerning the effects of sodium and potassium on the caulogenic response of *L. crithmoïdes* nodal explants, indicated that the addition of these cations at high concentration greatly improve the explant regeneration. Media supplemented with Na^+ at 50 mM (M7, M19) or 100 mM (M8, M20) with 1 or 50 mM of K^+, effectively promoted the shoot initiation. In fact, *L. crithmoïdes* is a halophyte which required high concentrations of Na^+ for a maximum growth (Zurayk and Baalbaki, 1996). It is able to grow at extremely high salinity equivalent to, at least, 200 mM NaCl (Flowers and Colmer, 2008) and its relative productivity decrease from the salinity of 110 mM (Lima *et al.*, 2021). Besides, according to Al Hassan *et al.* (2020), it can be found in a large range of soil salinities, and its frequency decreases as salinity reach values over 200 mM.

On the other hand, the addition of K^+ at the highest concentration (50 mM) without Na^+ allowed maximal shoot formation and elongation and enhance the number of leaves formed on the shoots (M17). In these cases, explants could have found an efficient and optimal potassium concentration to grow. In fact, when comparing *L. crithmoïdes* cultivated on different type of substrates, Grigore *et al.* (2012) found optimal growth on salt-free and nutrient-rich substrates, such as peat and garden soil. Potassium increases plants' nutrition efficiency, improves their growth, plant biomass productivity, and quality (El-Lethy *et al.*, 2013; Xu *et al.*, 2020; Taha *et al.,* 2020), in addition to its role in cell osmoregulation, regulation of enzymatic activities (Gerardeaux *et al.*, 2010; Jákli *et al.*, 2016), and protein synthesis requiring high K^+ concentration for tRNA fixing to ribosomes (Blaha *et al.*, 2000). Then, improving the nutritional status of plants in K^+ might be of great importance for the survival of crops under abiotic stress conditions, particularly salinity (Cakmak, 2005). According to Zurayk and Baalbaki (1996), *L. crithmoïdes* exhibited ionic relations typical of halophytes which can substitute Na^+ to K^+. Furthermore, maintaining a high K^+/Na^+ ratio is one of the adaptation strategies of halophytes to salinity (Assaha *et al.,*2017; Seleiman, 2019). Under high-salt conditions, halophytes undergo differential regulation of potassium and sodium ions and re-establishes Na^+/K^+ homeostasis (Kant *et al.*, 2010), including a reduction in Na^+ absorption and an increase in Na^+ compartmentalization (Yuan *et al.*, 2019).

However, the majority of *in vitro* plant culture investigations documented, focused on the growth regulators type and concentrations, which play a critical role in defining the plant cells and tissues development (Amiri and Mohammadi, 2021; Mitrofanova *et al.*, 2021). *In vitro* organogenesis studies of *Inula* species were

always induced by the hormonal balance. For *I. royleana* DC., the combination of 0.1 µM NAA (Naphthaleneacetic acid) and 5.0 µM kinetin was the most favourable for shoot proliferation (3.4 ± 1.2 and 5.1 ± 1.9 axillary shoots/node explant, with 100 and 94% shoot regeneration rates, respectively) (Stojakowska and Malarz, 2004). In our present work, we have reached 100% of caulogenesis with a higher average shoot number per node explant (8.3 ± 1.2) with adding 50 mM K^+ to the culture media. In fact, for Murthy *et al*. (2014), the optimum nutrient concentration is a critical determinant for the growth and morphogenesis of *in vitro* cultured tissues. For *Inula royleana*, the hormone combination of 5 mg/L BAP (Benzylaminopurine) and 2 mg/L IAA (Indole-3-acetic acid) regenerated 7.6 ± 0.49 shoots/leaf explant with 6.2 ± 0.28 cm mean length (Amin *et al*., 2018). For *I. verbascifolia* (Willd.) Hausskn. Perica *et al.* (2008) recorded a highest multiplication rate of 6.5 shoots/node explant on MS supplemented with 2.2 mM/L BAP and 2.9 mM/L GA3 (Gibberellic acid) and Thiem *et al.* (2003) induced indirect adventitious shoots on MS containing 0.88 µg/L BAP. Trejgell *et al*. (2018) obtained, on MS medium containing 1.0 mg/L BAP and 0.1 mg/L NAA, the highest caulogenesis rate (83.3%) on hypocotyl explant of *I. germanica* L., and the highest average number of shoots per shoot tip explant (12.0). Jabeen *et al*. (2007) reported that petiole explants of *I. racemosa* Hook. f. are the best responding explants. MS medium supplemented with BAP (0.25 mg/L) induced maximum number of rooted shoots (20.7 ± 0.8) directly on half strength MS medium free of hormones or with 1.0 mg/L IBA. More recently, Danova *et al.* (2021), demonstrated that the addition of BAP strongly stimulated axillary rosettes formation on shoot explant.

Effects of potassium and sodium ions on the adventitious rhizogenesis

Four parameters (%exaR, Nb.aR/ex, Nb.sR/aR, aRL) related to the rhizogenesis were used to evaluate the rhizogenic response for the four *L. crithmoïdes* explants (nodal, internodal, apical and basal leaf portions), cultured on the 20 culture media. Results are reported in Table 3. The statistical analysis of variance revealed a significant effect of the mineral balance and the explant type on the measured parameters. The analysis of the twenty tested media distribution according to their different effects in the plans defined by the PCA axes 1 and 2 (Figure 2A) and the HCA (Figure 2B) reveals three media groups. Each one has a different effect to promote the formation of adventives roots. Results revealed that nodal, apical and basal leaf explants have been found more suitable for enhancing adventitious roots than the internodal ones.

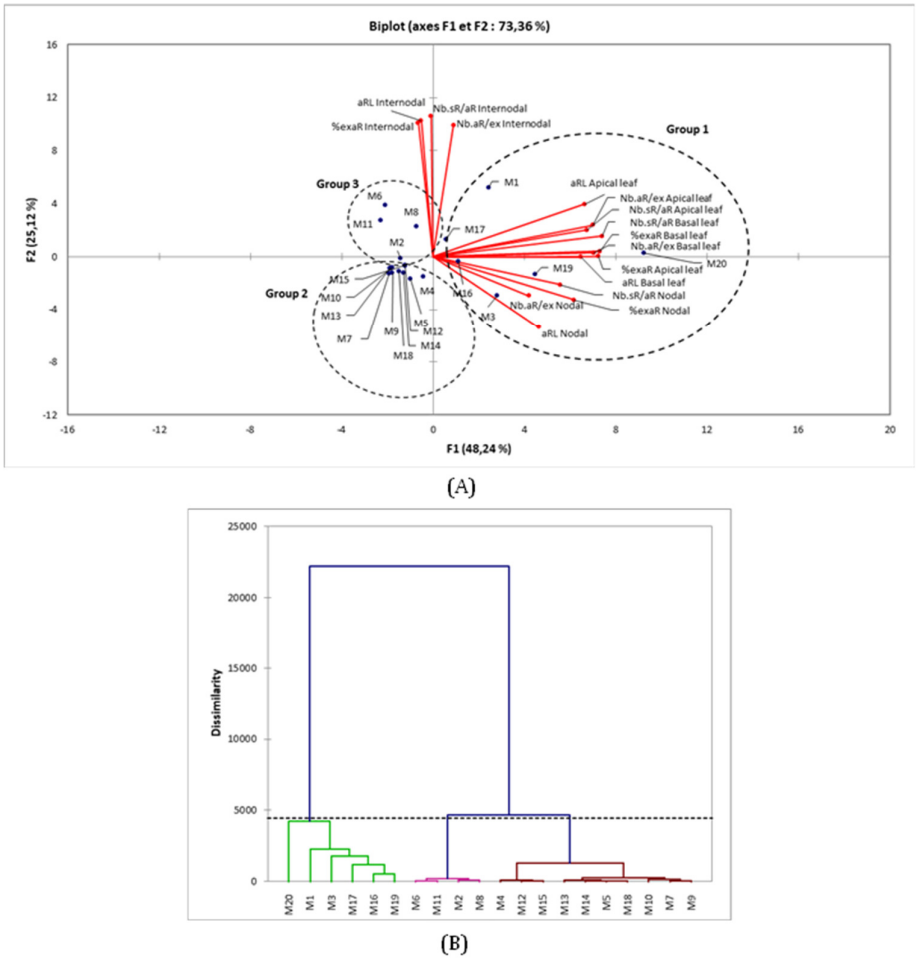

Figure 2. Distribution of the 20 media in the plans defined by the axis 1 and axis 2 of the PCA (A) and HCA (B) analyses, based on four rhizogenesis variables (%exaR, Nb.aR/ex, Nb.sR/aR and aRL) for *L. crithmoïdes* explants incubated in presence of K^+ and Na^+

The group 1 is formed by six media (M1, M3, M16, M17, M19 and M20). They are characterized by the best values of %exaR, aRL, Nb.aR/ex and Nb.sR/aR particularly on nodal, apical and basal leaf explants. With the basal leaf explant, we recorded the maximum values of %exaR (90.2 ± 1.3 and 96.1 ± 1.7%) on M19 and M20 media, characterized by the highest tested concentrations of potassium and sodium (50 mM K^+ and 50 mM Na^+; 50 mM K^+ and 100 mM Na^+, respectively). Then, the addition of high concentrations of NaCl effectively promoted roots neoformation. It was followed by the %exaR on the apical leaf (82.6 ± 0.1%; M20)

and on the nodal explants (86.0 ± 0.3, 78.3 ± 0.5 and 62.5 ± 0.6%) with M3 (0 mM K^+ and 50 mM Na^+), M20 and M19 media, respectively. For the internodal explants, adventive root neoformation percentage reached a maximum of 57.2% recorded in M1 medium free of K^+ and Na^+, and 40% with M2 and M11. Maximum average number of neoformed roots are recorded on basal leaf (8.5 ± 0.3; M19 and 9.3 ± 1.1; M20) and apical leaf explants (8.5 ± 0.2; M20). The average number of neoformed roots (7.9 ± 0.9) reached with the nodal explants cultured on M3 medium is interesting. In addition, we obtained the best Nb.sR/aR (8.4 ± 1.0 and 6.3 ± 0.3) with the basal and apical leaf explants respectively, recorded with M20 medium. The formation of the longest adventitious roots aRL reaching maximum values of 37.1 ± 0.7 and 39.8 ± 1.5 cm (M19 and M20, respectively), was recorded with the basal leaf explant followed by the nodal one (33.3 ± 0.5 cm; M3). With the M16 and M17 (10 mM K^+ and 100 mM Na^+; 50 mM K^+ and free of Na^+, respectively), rates and values of the rhizogenesis variables are quite less than those recorded with M3, M19 and M20 media, but still very interesting. In fact, with the basal leaf explants, %exaR reached 60.0 ± 0.3 and 65.0 ± 0.6% and the aRL 28.0 ± 0.5 and 29.3 ± 0.2 cm, with M16 and M17, respectively.

The group 2 is formed by ten media (M4, M5, M7, M9, M10, M12, M13, M14, M15 and M18) which allowed to achieve high values for the analysed variables, particularly with the nodal explants. In fact, the %exaR reached a maximum rate of 60% without sodium and in presence of 10 mM K^+ (M13), or in medium free of potassium and containing the highest concentration of Na^+ (100 mM). The rhizogenesis reaches interesting values with apical leaf explants incubated in M4 (100 mM Na^+ and free of K^+), M15 (50 mM Na^+ and 10 M K^+) and M12 (100 mM Na^+ and 5 mM K^+), 27.0, 26.0 and 27.3%, respectively. Nodal explants incubated on the media from this group 2 develop long adventive roots (25.4-21.3 cm). Media M2, M6, M8 and M11, are free or containing low concentrations of potassium (1 or 5 mM) regardless of Na concentrations and form the Group 3. The %exaR on the nodal explants, was reduced compared to this obtained with the media of the previous groups, but the value remain interesting (40.0-43.5%) with an important aRL varying from 22.5 to 24.7 cm. However, these media allowed an important development of adventitious roots (%exaR) on internodal explants (40.2-50.9), but root elongation, don't exceed 5 cm. Contrariwise, they are completely opposed to the leaf explants rhizogenesis.

Effects of potassium and sodium ions on the callogenesis induction

The evaluation of the callogenesis parameters (percentages of explants producing callus (%exCal), callus area (CalA) and fresh weight callus (FwCal) after two months of culture, reveals statistical differences in the intensity of the callus induction on the different explants (Table 4). PCA and HCA statistical analyses have defined three media groups (Figures 4 A and B). Group 1, formed by four media (M7, M8, M19 and M20), containing the highest tested concentrations of sodium; 50 mM (M7, M19) or 100 mM (M8, M20) in the presence of 1 mM K^+ (M7, M8) or 50 mM K^+ (M19, M20), allowed the maximum callogenesis induction with all the explants but at different intensities. The best callogenic response was observed on leaf explants; particularly on apical leaf ones, followed by internodal segments. Nodal explants showed the weakest callogenesis response.

On apical leaf explants, M20 medium; containing 50 mM K^+ and 100 mM Na^+, allowed the best callogenesis initiation compared to all the explants cultured on all tested media. The totality of the callogenesis parameters values is maximum. They reached 100.0 ± 0.0%, 740.0 ± 0.4 mm^2 and 853.0 ± 0.7 mg for %exCal, CalA and FwCal, respectively. The values recorded with the other three media (M7, M8 and M19) are slightly lower than those obtained with M20, but remained very high. In those media, the Na^+ is either in high concentration; 50 or 100 mM, and the K^+ is in a weak concentration 1 mM (M7 and M8, respectively), or Na^+ and K^+ are added in the same concentration equal to 50 mM (M19). On the other hand, basal leaf explants showed an efficient callogenesis response with these media, but less intense than that recorded with the apical leaf ones. Furthermore, the internodal segments are also suitable for callus induction of *L. crithmoïdes*. Their response is less important than that of leaf explants but better than nodal ones. For the nodal explants, the response to callogenesis is less intense than that recorded with the other explants (Figure 4).

Table 3. Effect of different combinations of K^+ and Na^+ on direct adventitious rhizogenesis from *L. crithmoïdes* explants *

Media	Explants															
	Nodal				Internodal				Apical leaf portion				Basal leaf portion			
	%exR	Nb.aR/ex	Nb.sR/aR.	aRL	%exR	Nb.aR/ex	Nb.sR/aR	aRL	%exR	N Nb.aR/ex	Nb.sR/aR	aRL	%exR	Nb.aR/ex	Nb.sR/aR	aRL
M1	58.3±0.6de	5.7±0.9ghi	0.9±0.8e	22.0±1.1hi	57.2±0.6a	2.7±0.5c	0.8±0.1a	4.0±0.1b	40.0±0.5a	5.0±1.8a	2.3±0.7a	6.0±0.7a	46.6±1.2c	4.0±1.3c	4.2±1.0b	5.0±1.2c
M2	40.0±1.5i	5.4±1.6ij	0.8±1.7e	23.4±1.4efgh	40.2±0.1h	0.1±0.6j	0.1±0.3e	0.1± .1h	9.3±0.4c	1.0±0.8d	0.2±0.7e	1.0±0.5d	19.3± 0.4d	2.0±1.8d	1.1±0.2c	1.1±0.1hi
M3	86.0±0.3a	7.9±0.9a	2.7±0.45a	33.3± 0.5a	40.0±0.1h	0.2±0.1i	0.1±0.2e	0.1± .0h	26.1±0.2e	1.3±1.2d	0.2±0.4e	0.8±0.1ef	52.0±0.2b	5.0± 1.3c	2.3±0.6c	3.6±0.0g
M4	60.0±1.9d	6.2±1.3ef	0.9±0.9e	24.5±0.8efg	3.9±0.4h	0.1± 0.1j	0.1±0.1e	0.1±0.1h	27.0±1.2e	1.5±1.0c	0.1±0.5f	0.5±0.2gh	12.0±1.2e	1.0±0.6d	1.9±0.4cd	1.3±0.2h
M5	50.0±1.7g	7.2±0.5b	1.7±0.6c	24.6±0.7efg	4.0±0.6 h	0.2 ± 0.1i	0.1±0.1e	0.1± .1h	9.0±0.1h	1.0±0.3d	0.1±0.1f	0.3±0.7hi	5.1± 0.9g	0.5±0.3e	0.21±0.16f	0.1±0.1hi
M6	40.0± 1.1i	6.0±0.9fg	0.8±1.5e	24.7±1.4efg	50.9±0.7b	2.2±1.6d	0.8±0.3a	5.0±0.9a	0.5± 0.1i	0.2±0.6f	0.1± 0.1f	0.2±0.4 ij	5.1± 0.1g	0.5±0.7e	0.3±0.2e	0.1±0.3hi
M7	40.0± 1.6i	6.5±1.5de	1.0±1.8de	24.0±1.9efgh	10.0±0.6d	0.1±0.3j	0.1±0.6e	0.2±0.5g	0.9± 0.1hi	0.15±0.3f	0.4±0.2d	0.2± 0.0ij	1.0±0.2h	0.5± 0.1e	0.2±0.7f	0.1± .2hi
M8	40.0± 1.7i	6.7±1.2cd	1.85±0.23c	24.4±2.5efg	50.5±0.8b	2.7±1.3c	0.4±0.4c	2.0±0.2d	1.0±0.2h	0.9±0.4d	0.4±0.2d	0.2±0.1ij	16.0± 0.8f	2.0±1.3d	2.3±0.5e	1.2±.3hi
M9	40.0± 1.9i	5.9±0.8fgh	0.5±0.4f	24.7±1.0def	7.0±0.2g	0.11±0.1j	0.1±0.2e	0.1±0.3h	8.0± 0.4 g	1.0±0.8d	0.2±0.5e	0.5±0.3gh	5.2± 0.4g	1.0±0.4d	0.1±0.6g	0.8±0.2efg
M10	40.0±1.8i	5.7±0.8hi	0.5±0.3f	21.3±0.7fghi	7.1±0.1g	0.2±0.5i	0.1±0.2e	0.1±0.4h	1.0±0.3h	0.5±0.8e	0.2±0.6e	0.2±0.2ij	16.0±0.6e	2.0±0.3d	2.1±0.3e	1.6± 0.2g
M11	43.5±1.7h	5.3±1.0jk	0.5±0.08f	22.5±0.7fghi	40.3±1.5c	2.0±0.7b	0.5±0.5b	3.5±0.6c	0.5±0.4i	0.2±0.3f	0.2±1.0e	0.2±0.0ij	5.1±0.3g	1.0±0.1d	0.3±0.8e	0.7±0.2fg
M12	53.0±1.2f	5.0±1.4kl	0.2±0.3h	25.4±1.6de	7.0±1.01g	0.5±0.1h	0.2±0.4d	0.2± 0.1g	27.3± 0.3d	2.0±0.3c	0.4±0.2d	0.6±0.1fg	6.0±0.3g	0.5±0.4e	0.1±0.4g	0.6± 0.1g
M13	60.0±1.3d	5.1±0.3jk	0.2±0.3gh	24.8±0.4def	8.0±0.9f	0.5±0.7h	0.2±0.9d	0.2±0.1g	1.0±0.1h	0.5±0.1e	0.2±0.1e	0.2± 0.0ij	5.2± 0.9g	0.5±0.43e	0.1±0.1g	0.8±0.2efg
M14	50.0±0.8g	5.7±0.6ghi	0.5±0.2f	24.6±0.9efg	4.6±1.05h	0.2±0.2i	0.1±0.7e	0.2±0.2g	3.0±0.1f	0.5±1.0e	0.1±0.12f	0.4±0.2ghi	13±1.2f	1.6±0.3d	2.2±0.2f	2.4±0.2d
M15	50.0±0.7g	5.2±1.2jk	0.3±0.05g	25.3±1.5de	7.6±0.8ef	0.7±0.2g	0.2±0.1d	0.2±0.5g	26.0±0.6f	1.0± 0.1d	0.1±0.5f	0.9±0.2de	11.0±0.6f	1.5±0.1d	0.3±0.4e	0.9±0.2ef
M16	56.3±0.6e	5.1±0.8jk	0.5±0.7 f	24.4±0.3efg	10.2±0.2d	0.8±1.0f	0.2±0.5d	0.2±0.1h	29.0±0.9c	1.0± 0.1d	0.2±0.2e	1.0±0.5d	60.0±0.3b	6.3±0.2c	3.3±0.2e	28.0± 0.5b
M17	50.0±0.1g	4.7±1.6l	0.3± 0.5g	22.6±1.4ghi	40.8±1.0c	0.5±0.01h	0.4±0.2c	1.0± 2.9e	8.3± 0.4d	1.0± 0.2d	0.1±0.2f	1.0±0.2d	65.0±0.6b	7.4±0.2c	4.2± 0.8f	29.3±0.2e
M18	50.0±0.7g	5.3±0.8jk	1.2± 1.2d	25.5±0.6cd	10.0±0.2d	0.5±0.2h	0.1±0.5e	0.1±0.1h	1.0± 0.4f	0.5±0.9e	0.4±0.2d	0.6±0.2fg	5.0±0.8g	1±0.01d	0.3±0.1e	0.6±0.02g
M19	62.5±0.6c	7.0±1.6bc	2.5±0.9ab	29.2±1.78b	9.1±0.9e	0.8±0.6f	0.1±0.2e	0.2±0.9g	35.0± 1.4b	2.2±0.5bc	0.9±0.3bc	1.9±0.4c	90.2±1.3a	8.5±0.3b	5.9±0.1b	37.1±0.7a
M20	78.3±0.5b	7.3±1b	2.5±0.3b	28.7±0.7bc	9.5±0.0de	1.0±0.8de	0.2±0.7d	0.6±0.3f	82.6± 0.1a	8.5±0.2b	6.3±0.3b	7.1±1.2b	96.1± 1.7a	9.3±1.1a	8.4±1.0a	39.8± 1.5a

* Note: Data represents the mean of three replicates. The means ± the standard deviations followed by the same letter within the same column are not significantly different ($p > 0.05$) (Tukey's test). Culture duration was 2 months.

%exaR; Percentage of explants that initiated adventive roots, Nb.aR/ex; Number of adventive roots per explant, Nb.sR/aR; Number of secondary roots per adventive root, aRL; Adventive root length (cm)

ex; explant, explants are: Nodal, Internodal, Apical leaf portion, Basal leaf portion

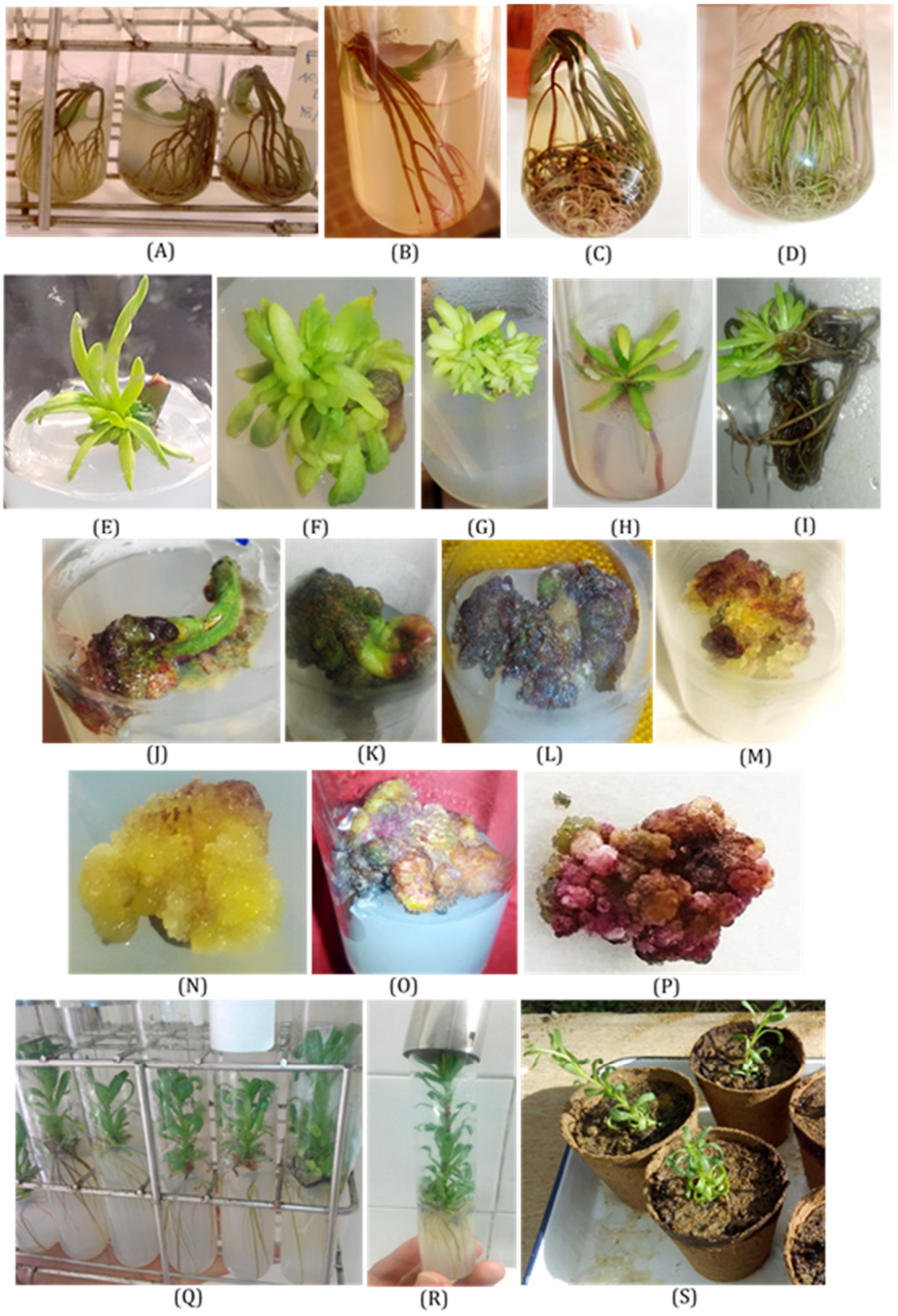

Figure 3. *In vitro* growth responses of *Limbarda crithmoïdes* explants in optimum conditions; (A-D) Adventitious roots initiation on apical and basal leaf portions; (E-H) Very short axillaries formed on the nodal segments formed on the nodal segments; (I) Rhizogenesis from nodal explants; Callus formation (J-L) from basal and apical leaf portions, (M, N) nodal and (O, P) internodal explants; (Q, R) *In vitro* rooted plants; (S) Acclimatized regenerated plants

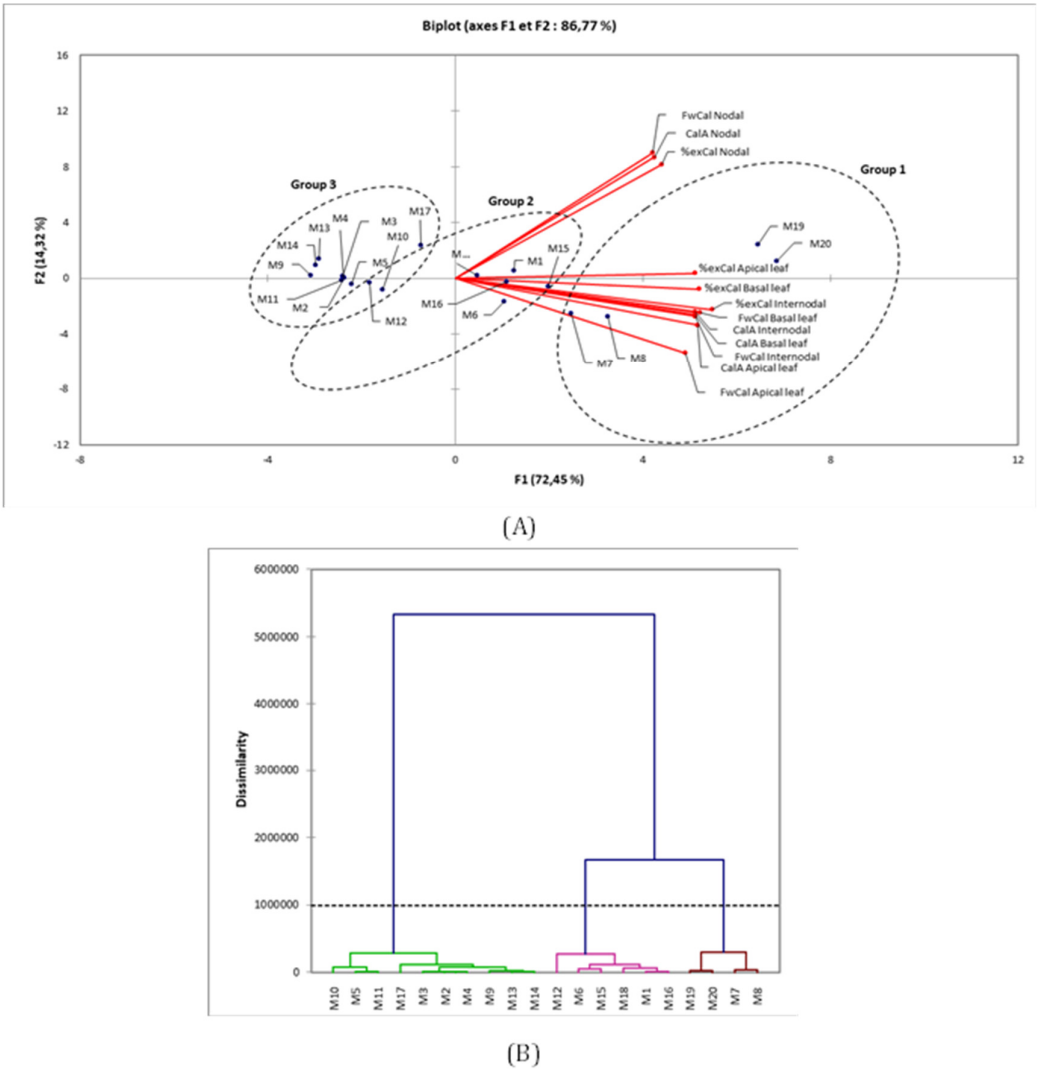

Figure 4. Distribution of the 20 media in the plans defined by the axis 1 and axis 2 of the PCA (A) and HCA (B) analyses based on three callogenesis variables (%exCal, CalA and FwCal) for *L. crithmoïdes* explants incubated in presence of K^+ and Na^+

Therefore, supplementing high concentrations of sodium and potassium induced a remarkable callus formation. In fact, callus culture, the most essential step to initiate an *in vitro* culture, requires an optimum supply of nutrients for sufficient growth (Bhatia *et al.*, 2015). Indeed, according to Manaf (2008), the variable ratios of K^+/Na^+ can disrupt various enzymatic processes in the cytoplasm, which leads to callus neoformation; anarchic division of cells that cannot be organized into a well-differentiated tissue. Moreover, nutrient deficiency or imbalance can cause abnormal physiological responses in plant cultures such as hyperhydricity, callus formation and necrosis (Teixeira da Silva *et al.,* 2020; Doungous *et al.,* 2022).

Group 2 formed by six media (M1, M6, M12, M15, M16 and M18) also promote callogenesis but less intense than that obtained with the group 1. The highest callogenesis rate was registered on apical leaf portion (70.0 ± 0.5%) cultured on M16, which contain 10 mM K^+ and 100 mM Na^+. On the same latest medium, we obtained the best CalA and FwCal on internodal explants (580 ± 0.3 mm^2 and 784 ± 0.5 mg, respectively). The M12 stands out from the group it induced a weak callogenesis compared to others.

Table 4. Effect of different combinations of K^+ and Na^+ on callogenesis of *L. crithmoïdes* explants after 2 months of culture

Media	Nodal			Internodal			Apical leaf portion			Basal leaf portion		
	%exCal	CalA	FwCal	%exCal	CalA	FwCal	%exCal	CalA	FwCal	%exCal	CalA	FwCal
M1	15.1±0.2c	70.3±0.5g	173±0.2d	50.1±0.3d	478±0.5f	639±0.1g	50.0±0.7f	59.4±0.7k	335.8±0.8k	59±0.1d	214.2±0.2i	374.5±0.1h
M2	0.5±0.6hi	3±0.1j	1±0.2jk	5.2±0.1h	5.1±0.3kl	3.0±0.1kl	40.0±0.3g	55.3±0.5l	287.3±0.4l	40±0.6g	50±0.4q	110.0±0.6q
M3	0.6± 0.4hi	2.5±0.1j	0.8±0.7jkl	3.2±0.7ij	1.6±0.7m	1.5±0.3lm	50.0±0.2f	48.3±0.7m	211±0.0o	40±0.7g	42±0.6r	97.2± 0.5r
M4	1.2±0.3h	3.5±0.8j	1.2±0.3	0.5±0.2k	1.0±0.2m	0.6±0.3m	40.0±0.2g	55.2±0.9l	256±0.6n	40±0.0g	60±0.8p	140.0±0.2o
M5	0.1± 0.1i	1±0.7k	0.2±0.2m	0.3±0.2k	1.0±0.1m	0.4±0.2m	10.0±0.3j	75±0.6j	350.5±0.7j	20±0.7h	256.6±0.2h	320.0±0.4k
M6	0.1± 0.1i	1±0.9k	0.2±0.1m	50.8±0.8d	436±0.5i	602±0.2i	60.0±0.1e	260.0±0.2g	533.0±0.1e	60±0.2e	315±0.4f	420±0.8f
M7	0.1± 0.2i	1±0.1k	0.2±0.5m	50.1±0.7d	444±0.1h	620±0.8h	70.0±0.8d	680.3±0.4b	744.7±0.6b	69.0±0.1c	400.5±0.2d	540.8±0.9d
M8	0.1± 0.5i	1±0.3k	0.2±0.5m	67.8±0.2c	574±0.3d	760±0.6d	80.0±0.3c	630±0.1c	810.0±0.0c	80.0±0.2b	419.1±1.6c	650±0.7c
M9	0.4±0.7hi	1.2±0.4k	0.3±0.4lm	4.1±0.3hi	3.0±0.3lm	2.0±0.3klm	10.1±0.5j	20.1±0.9n	80±0.7p	10±0.1i	113±0.4m	200±0.8n
M10	0.2± 0.2hi	1±0.1k	0.1±0.3m	5.5±0.9h	6.0±0.7k	4.0±0.1k	20.0±0.4i	277.0±0.6e	496.6±0.8f	10±0.7i	301.3±0.6g	400 ±0.1g
M11	0.2±0.7hi	1±0.6k	0.1±0.2klm	4.2±0.1hi	3.2±0.4lm	3.7±0.6k	30.0±0.4h	47.5±0.1m	261±0.4m	10±0.6i	164±0.5k	285±0.5l
M12	0.2± 0.4hi	1±0.5k	0.2±0.1m	30.2±0.3f	412±0.8j	396.8±0.2j	20.0±0.7i	15±0.4p	22.4±0.3t	10±0.3i	145±0.3l	250.5±0.2m
M13	6.5±0.7f	74.8±0.2f	82.1±0.1g	2.1±0.1jk	1.0±0.1m	0.8±0.1m	10.0±0.6j	17.3±0.9o	28.3±0.1s	10±0.5i	70.0±0.7n	90.1±0.1s
M14	4.8±0.1g	20±0.5i	50±0.4i	2.0±0.1jk	1.0±0.1m	0.8±0.1m	30.0±0.6h	17.5±0.3o	37±0.2q	20±0.1h	35.0±0.8s	30±0.3t
M15	6.2±0.4f	92.5±0.6d	112±0.4e	60.6±0.7c	470.0±0.4g	655.0±0.4f	60.0±0.1e	265.0±0.1f	480.0±0.0g	40±0.4g	344.4±0.6e	494.4±0.5e
M16	7.6±0.9e	68.0±0.4h	78±0.0h	50.1±0.1d	580.0±0.3c	784.0±0.5c	70.0±0.5d	180.0±0.8h	380.0±0.2i	50±0.7f	211.4±0.5j	368.9±0.4i
M17	14.1 ±0.5c	129.6±0.2c	217±0.1c	2.4±0.1ij	1.3±0.2m	1.0±0.6lm	70.0±0.2d	17.1±1.1o	35.0±0.0r	40±0.8g	165±0.4k	333.2±0.2j
M18	8.3±0.6d	80.0±0.6e	94.6±0.0f	40.2±0.3e	523.0±0.9e	721.6±0.7e	60.0±0.7e	176.0±0.7i	400.0±0.4h	50±0.6f	64.9±0.1	136.0±0.7p
M19	41.6±0.1b	319.0±0.3a	412±0.1a	76.3±0.4b	645±0.9b	806.0±0.1b	90.5±0.2b	690.5±0.3d	843.0±0.3d	88.9±0.4a	480.9±0.1b	666.0±0.1b
M20	43.8±0.3a	226.0 ±0.1b	344±0.6b	82.1±0.6a	659.0±0.9a	846.0±0.3a	100.0±0.0a	740.0±0.4a	853.0±0.7a	89.0± 0.3a	572.6±0.2a	785.0±0.1a

***** Note: Data represents the mean of three replicates. The means ± the standard deviations followed by the same letter within the same column are not significantly different ($p > 0.05$) (Tukey's test). Culture duration was 2 months.

%exCal; Percentage of explants producing callus, CalA; Callus area (mm^2), Fw.Cal; Fresh weight of callus (mg)

Group 3 formed by ten media (M2, M3, M4, M5, M9, M10, M11, M13, M14 and M17) was characterized by a poorer callogenic performance, except with M17 (50 mM K^+ and free of Na) which allowed the initiation of 70.0% of calli on the apical leaf explants and an interesting proliferation of callus cells on internodal segments (165 ± 0.4 mm^2 and 333.2 ± 0.2 mg for CalA and and FwCal, respectively). The others media of this group, allowed the lowest values of all the callogenesis parameters. They can be considered as totally opposed to the callus initiation.

Published works on the callogenesis induction of *L. crithmoïdes* without the addition of hormones are to our knowledge absent. However, callus initiation was earlier induced by adding hormones (Bucchini *et al.*, 2013) for the same species from Italy. They noted a maximum callogenesis rate (90.2%) on the leaf explants grown on MS basal medium supplemented with 1 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid). This result was close to the maximum percentages of %exCal obtained in our study on the apical (90.5% and 100%) and basal (90%) leaf explants, in presence of maximum concentrations of potassium and sodium (M19 and M20). For *I. royleana* the combination of 5 mg/l BAP and 2 mg/l IAA, induced maximum callus (100%) (Amin *et al*., 2013). From culture callus obtained on *I. royleana* leaf explants, Amin *et al*. (2017) produced 33.5 and 12.8 shoots/callus in MS medium supplemented with 1 mg/L BAP and 0.8 mg/l kinetin, respectively. To initiate callus proliferation, leaf explants of *I. helenium* L. are inoculated onto MS medium supplemented with 2,4-D (4.52 µM/l) and kinetin (1.39 µM/L) (Stojakowska *et al.*, 2016). MS medium with 1.2 mg/L 2,4-D was found to be the best for callus induction using rhizomes as explants in *I. japonica* Thunb. (Yong-Mei *et al.*, 2008). Moreover, for *I. Britannica* L., Zhou (2010) induced callus from leaves and roots explants. Maximal rate of callogenesis (100%) was obtained in MS medium without TDZ (Thidiazuron) from leaves, and with 0.05 mg/1 TDZ from roots.

Generally, callogenesis induction without the addition of hormones is scarce. In Tunisia, for *Prosopis farcta*; halophyte and native species, Harzallah-Skhiri (2003) and Stambouli *et al.* (2011, 2012) revealed that, media containing maximum concentrations of sodium (100, 150 mM) with 0.1 and 20 mM of calcium, or 0.1 mM sodium with 25 mM of sulfate ion promote optimal callus formation on hypocotyl (36 and 84%, respectively). Previously, in Algeria, Benrebiha *et al.* (1992) proved that in the absence of growth substances, callus control is possible by addition of macronutrients in the *in vitro* culture medium of *Atriplex halimus* L. They explained that their role is at the cell membrane level through an indirect effect on the internal hormonal balances. Other research works focused on the selection of the basal salt composition to optimize the nutrient component for inducing *in vitro* morphogenesis of several species. We can name among them Niedz and Evens (2007) who demonstrated that the growth of *Citrus sinensis* (L.) non-embryogenic callus could be regulated and controlled *via* the mineral nutrient components of the medium. For *Corylus avellana* L., Akin *et al*. (2017) tested the effect of Ca^{2+}, Mg^{2+}, K^+, NH_4^+, NO_3^- and SO_4^{2-} ions and give the best concentrations, to be added in media, allowing the best callus formation. More recently, the study of Elyazid *et al.* (2021) optimized the concentration of $CuSO_4$ in banana tissue culture media to improve the growth and development of micropropagated banana plants by enhancing shooting and rooting. A concentration of 0.025 mg/L of copper sulphate was optimal for the maximum shoot number and length. The root length of banana plantlets was significantly enhanced at 30 mg/l of $CuSO_4$. A global review on this subject was presented by Ramage and Williams (2002).

Optimal media content allowing the best organogenesis and/or callus initiation responses

Six optimal *in vitro* culture media showing the best values of the various organogenesis expressions and callus initiation, corresponding to the most efficient explant type are selected (Table 5).

Media containing maximum concentrations of K^+ (50 mM) in presence of the highest concentrations of Na^+ (50 and 100 mM) (M19 and M20, respectively) favoured the best root neoformation on the basal and apical leaf explants. The caulogenesis has been registered only on the nodal segment explants. The shoot

regeneration was maximum in presence of 50 mM of K^+ (M17). It was followed by media containing the highest concentrations of these two cations (M19 and M20), and then by adding 1 mM of K^+ to 50 mM Na^+, or to 100 mM Na^+ (M7 and M8, respectively). All the six selected media gave maximum performance towards callogenesis. However, the best callus induction was recorded with apical leaf portions cultured on media supplemented with 50 mM K^+ and 100 mM Na^+ (M20) or with 50 mM K^+ and 50 mM Na^+ (M19).

Table 5. Identification of media promoting the best organogenesis and/or callus initiation responses of *L. crithmoïdes* cultured explants

Media	Concentration (mM)		Explant type	Root neoformation and/or shoot regeneration and/or callus initiation										
	K^+	Na^+		%exaR	Nb.aR /ex	aRL (cm)	%exS	NbS /ex	SL (cm)	Nb.e /ex	leL (cm)	%exCal	CalA (mm^2)	FwCal (mg)
M7	1	50	Apical leaf									70.0		
			Basal leaf									69.0		
			Nodal				60.1			58.2				
M8	1	100	Apical leaf									80.0		
			Basal leaf									80.0		
			Nodal				61.2			66.2				
M16	10	100	Apical leaf									70.0		
			Internodal										580.0	784.0
M17	50	0	Nodal				100	8.3	10.1	70.6	5.1			
			Apical leaf									70.0		
M19	50	50	Basal leaf	90.2	8.5	37.1						89.9		
			Apical leaf									90.5	680.3	
			Nodal				70.6			66.5				
			Internodal									76.3	645.0	806.0
M20	50	100	Basal leaf	96.1	9.3	39.8						90.0		
			Apical leaf	82.6	8.5							100.0	740.0	853.0
			Nodal				80.0			68.9				
			Internodal									82.1	659.0	846.0

Acclimatization

After 3 months in the greenhouse, 93% of the propagated rooted plants from nodal segments in optimum growth conditions, survived (Figures 3Q-S). The *in vitro* generated calli have a compact texture and are yellowish to brownish in colour can indicate an accumulation of polyphenols (Figures 3L-P). Thus, they can be used for the extraction of these bioactive molecules and for the evaluation of several activities of their extracts.

Conclusions

Based on the performed analysis, in the present research work, we demonstrated that the *in vitro* growth, the organogenesis and/or the callogenesis of *L. crithmoïdes* can be induced, without the use of growth hormones, but only by modification of the media mineral composition. We were able to define several model media prepared from MS medium containing Na^+ and K^+ ions allowing a maximum shoot regeneration, root neoformation and induction of callus. This effective propagation procedure would add valuable information to the existing knowledge for the micropropagation protocols of *Inula* species and other halophytes, and will allow continuous production of a sufficient quantity of plant material for its use in ornamentation as well as for medicinal purposes by the extraction of its potential bioactive compounds.

Authors' Contributions

HB performed the experiments, statistically analysed the data and wrote the draft of the manuscript; SK monitored the cultures and regularly recorded the results; KH provided the plant material; MD took and described the photographs; SSE supervised the experiments, wrote and discussed results and FHS revised the manuscript. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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