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IN VITRO MULTIPLICATION AND ACCLIMATIZATION OF THE ROYAL FERN, *OSMUNDA REGALIS* L.

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Abstract

Osmunda regalis L. *(royal fern) is a species with special ornamental values that can reach 2.5 m in height. The species is extinct in Romania and threatened in several European countries, it prefers humid habitats with spores having a short viability period. . Our studies focused on the introduction of this species in in vitro culture, in order to propagate the sporophytes and subsequent acclimatization. The spores were sterilized and inoculated on the nutrient medium MS ½, the differentiation of the protalli being achieved after approximately 3 weeks from the initiation of the culture. Sexual multiplication was achieved by differentiating sporophytes in gametophytic cultures on nutrient depleted medium MS½ without ammonium nitrate and vitamins. Later, clonal multiplication was achieved by detaching the sporophytes and subcultivation on the same type of medium. Acclimatization was carried out on seven substrate variants, with different pH, composition and granulation, the best results being obtained on the substrate which contained 80% peat and 20% humus. Through this technique, it was possible to obtain viable sporophytes, also, rooting and acclimatization at the greenhouse level for horticultural exploitation or the repopulation of suitable habitats.*

Key words*: Osmunda regalis, fern, gametophyte, sporophyte.*

INTRODUCTION

Osmunda regalis L., royal fern, is a species of Pteridophyta from Osmundaceae family, one of the oldest families of ferns, whose fossils date back over 200 million years to the Permian (Smith et al., 2006; Hewitson, 1962). The name reflects its imposing and elegant appearance. It is a perennial, isospore, with a vigorous, strongly branched oblique rhizome (Săvulescu, 1952). The fronds are double-pinnate, arranged in a dense, majestic crown that can reach 2.5 m in height (https://www.rhs.org.uk/plants/trials-awards).

The species *O. regalis* was valorised in horticulture and landscaping due to its appearance; its spectacular foliage in autumn acquires shades of red and brown, creating a visual pleasant aspect. The Royal Horticultural Society awarded the species *Osmunda regalis* by giving the prestigious Award of Garden Merit for outstanding horticultural characteristics (https://www.rhs.org.uk/plants/trials-awards).

The rhizome of this fern has been used as a substrate for growing orchids and epiphytes, and in Russia and Europe, it is used for horticultural purposes (Matchutadze, 2014; https://www.missouribotanicalgarden.org; https://herbaria.plants.ox.ac.uk)

Although not recorded in official pharmacopeias, this species has a long history of use in traditional medicine. The roots and leaves of the *O. regalis* species have been used to treat jaundice, rheumatic pains, bone fractures, wounds (Matchutadze, 2014). In some regions of Spain, it is rare and is classified as endangered due to its harvesting for medicinal purposes. A macerate with white wine (antojil wine) is prepared from the middle part of the rhizome and is used as a remedy for digestive disorders (Molina et al., 2009).

The decrease in precipitation and the expansion of drainages contribute to the reduction of habitat and essential resources for this species, increasing the risk of extinction.

According to the IUCN assessments, 2014 the species *O. regalis* is critically endangered in Belarus, Bulgaria, Croatia, Hungary, Iran and Luxembourg, vulnerable in Germany, Switzerland and Poland (Gdula et al. 2014) and rare in Norway (Birks et al., 1991).

In Romania, *O. regalis* is mentioned as an extinct species in two Red Lists (Boșcaiu et al., 1994; Dihoru & Dihoru., 1994).
Under typical storage conditions, the spores of the species *O. regalis* showed reduced viability (Stokey, 1951; Windham et al. 1986).
O. regalis has a long life cycle, of almost two years, from spore sowing to the first production of sporophytes and spores (Klekowski, 1967).
In the current context of anthropogenic changes and pressures on biodiversity, taking into account the specific ecological characteristics of the species, which includes a preference for humid environments and a long-life cycle, *in vitro* micropropagation and the acclimatization process represent an alternative for the revitalization of the *O. regalis* species. Recent studies on extracts obtained from this plant have identified some of the bioactive compounds responsible for the beneficial effects (Mitoi et al., 2024) and analysed the impact of osmunda extracts on cancer cells, demonstrating their potential use as anticancer remedies (Schmidt et al., 2017; Carpinteyro Diaz et al., 2024).
The distribution area of the species includes temperate and tropical regions. It prefers moist and shady habitats, growing in peatlands, swamps, lake shores, and forests, on moist and peaty mineral soils (Birks et al., 1991; Matchutadze, 2014).
The aim of this study was to identify an efficient method of multiplication and acclimatization using *in vitro* culture technique for the species *O. regalis*. The objective was to obtain a large number of sporophytes through sexual reproduction *in vitro* and vegetative multiplication of the obtained sporophytes, as well as to determine an adequate substrate that would meet the specific nutritional requirements of this pteridophyte, to ensure a high survival rate upon acclimatization to greenhouse conditions.

MATERIALS AND METHODS

Plant material

The biological material used to initiate *in vitro* culture was represented by spores harvested from plants grown in the Dimitrie Brândză Botanical Garden from Bucharest. The spores were preserved at 4°C until use.

Obtaining the gametophytic cultures from spores

For sterilization, the spores were introduced in filter paper envelopes and immersed in a 3% H_2O_2 solution or in a mixture of 6% $CaCl_2O_2$ with Tween 20 for 7-10 minutes, followed by washing three times with sterile distilled water.
The spores were inoculated in Petri dishes on MS basal medium (Murashige and Skoog, 1962) with half concentrations of micro and macroelements, supplemented with vitamins B5 (Gamborg et al., 1968) and 30 g/L sucrose, at an adjusted pH of 5.8 (MS ½).
The culture vessels were maintained in growth chambers at a temperature of 22-24 ±2°C, with a photoperiod of 16 hours at a fluorescent intensity of 40.5 µmol/m^2s.
Gametophytes obtained from spore germination were subcutivated at each 2-month, on the same type of nutrient medium MS ½.

Obtaining sporophytes from gametophytes by sexual reproduction

For sporophytes differentiation from gametophytes, 1 g of mature gametophyte was transferred to Erlenmeyer flask on MS ½ solid nutrient medium with or without ammonium nitrate and vitamins (Makovski et al., 2016). The three experimental variants were:

1) MS ½ solid;
2) MS ½ solid without ammonium nitrate and vitamins- MFAV;
3) MFAV, to which 1 ml of same liquid medium was added to the gametophytes surface.

Sporophytes cultures were inoculated in 250 ml Erlenmeyer flasks containing 100 ml of nutrient medium and incubated under the same growth conditions mentioned above. For each nutrient medium variant, 6 repetitions were performed. The number of sporophytes was evaluated at 4, 5 and 6 months after inoculation.

Evaluation of sporophyte leaves and roots growth

Sprophytes obtained on MFAV were detached from the gametophyte and inoculated on four culture medium variants:

1) MS ½ solid;
2) MFAV solid;

3) MSR- MS nutrient medium supplemented with 1 mg/l benzylaminopurine (BAP), 1 mg/l kinetin (K), 1 mg/l 1- naphthalenacetic acid (ANA);
4) E7- MS nutrient medium supplemented with 1.8 mg/l indolilacetic acid (AIA), 0.02 mg/l K.

The cultures were incubated in growth chambers at a temperature of 22-24±2°C, with a photoperiod of 16 hours. After 3 months from inoculation, the sporophytes were weighed and measured, by registration of the following morphometric parameters: sporophyte growth rate (SGR), leaf number (LN), the largest leaf length (LLL), the smallest leaf length (SLL), rootlets number (RN), the longest rootlet length (LRL), the smallest rootlet length (SRL).

Multiplication of sporophytes

Vegetative multiplication was achieved by sectioning the sporophytes in half at the rhizome level and inoculating them on fresh MFAV medium. The sporophyte cultures were grown in 100 and 250 ml Erlenmeyer flasks or vessels with 50 and 100 ml medium under the same controlled conditions mentioned above.

One year after the experiment started, the following gravimetric and morphometric parameters were determined: sporophyte growth rate (SGR), leaf number (LN), the largest leaf length (LLL), the smallest leaf length (SLL), the longest rootlet length (LRL).

Obtaining gametophyte from leaves and rootlets fragments

Leaves and rootlets from juvenile sporophytes were detached or sectioned using a sterile scalpel and cultured on MFAV medium. After 20 days from inoculation, the gametophyte began to develop.

Acclimatization of sporophytes to soil in pots

In a first experiment for acclimatization, 1-year-old sporophytes were used and three substrates variants were tested (Table 1):

- substrate for indoor plants (S1);
- substrate for sowing and propagation with 80% peat and 20% humus (S2);
- -substrate for ornamental shrubs with bark enriched with basic nutrients and trace elements B, Cu, Mn, Mo, Zn, Fe (S3). Additionally, for this experiment the sterilized variants of these substrates were used, SS1, SS2 and SS3.

The plant growth process was monitored over a period of one year. The assessments were carried out in four stages: at the beginning of acclimatization, at one month, three months and at one year of potting plants. The analyzed parameters included: sporophyte growth rate (SGR), leaf number (LN), the largest leaf length (LLL), the smallest leaf length (SLL), root length (RL), radicular volume (RV), survival percent of sporophytes (SP).

In the second acclimatization experiment, four other four types of substrates were tested:

- aquatic plants substrate with peat, quality compost, sand, clay (S4);
- acidophilous plants substrate with horticultural peat (S5);
- cacti substrate (S6);
- natural peat substrate (S7).

Also, the sterilized variants SS4, SS5, SS6 and SS7 were used.

Plant growth was monitored one month after the sporophytes transfer to the pots. The assessments included the same biometric parameters analysed as in the first acclimatization experiment.

All types of substrate except variant 7 were purchased from Agro CS Romania SRL. In both experiments, both sterile (SS1, SS2, SS3, SS4, SS5, SS6 and SS7) and non-sterile (S1, S2, S3, S4, S5, S5 and S7) substrates were tested, differentiated according to pH, composition and conductivity (Table 1).

Table 1. Some physicochemical parameters of the used substrates.

Substrate	pH	N (mg/l)	P_2O_5 (mg/l)	K_2O (mg/l)	Conductivity ($mS.cm^{-1}$)
S1	4.7	150-200	100-200	200-300	0.8
S2	5.5	300	100	300	0.8
S3	5.5	50-250	80-250	100-300	0,8
S4	6.7	100-200	80-200	100-300	0.8
S5	4.5				0,5
S6	4.6	20-150	30-150	40-200	0,5
S7	4.2				

Planting was carried out in plastic seedling pots using the same amount of substrate. Each experimental variant had 6 repetitions.

Acclimatization was carried out under natural lighting conditions, at a temperature of 22 °C, and humidity of 40-45%. To ensure a humidity level similar to that of *in vitro* cultivation conditions (approximately 80%), the plants were covered with glass pots. Adaptation to

greenhouse environmental conditions was achieved gradually. The plants were uncovered daily for 10 minutes, with exposure to ambient atmospheric conditions gradually increasing until complete acclimatization was achieved.
The gravimetric and morphometric parameters used for all the experiments were obtained as follows:

- growth rate = final weight – initial weight/initial weight;
- roots and leaves length were measured with a ruller and calculated as difference between initial and final value;
- total root volume, determined by the volumetric method.

All gravimetric measurements were made on the adwag AS 220/C/2 balance (Radwag, Radom, Poland)

RESULTS AND DISCUSSIONS

The life cycle of *O. regalis* fern is characterized by alternation of generations, which includes a haploid gametophytic phase, involved in the production of gametes, and a diploid sporophytic phase responsible for sporogenesis. The sporophytic phase is dominant, characterized by morpho-functionally differentiated roots, rhizomes, and fronds, including specialized leaves (sporophylls) that contain sporangia, where meiosis and the formation of haploid spores occur.
Under our experimental conditions of *in vitro* culture, involving aseptic spores germination, prothalli were differentiated approximately three weeks after inoculation (Figure 1a) . The obtained gametophytic culture was subsequently cultured for 2 months on MS½ medium (Figure 1b). This gametophytic culture was multiplied and maintained in long-term *in vitro* conditions for several years.

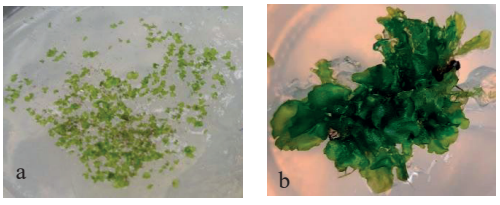

Figure 1. Gametophytes cultures of *O. regalis*: a- primary protalli culture and b- gametophyte culture

Evaluation of the gametophyte growth rate
Initially, the growth of the gametophyte culture was carried out on half strength MS medium (MS½), because our previous studies on other fern species showed that this nutritive medium proved to be the most advantageous (Aldea et al., 2016). Subsequently, a better gametophyte development was observed by using the solid MS½ medium without NH_4NO_3 and vitamins (MFAV) in order to obtain sporophyte differentiation. Evaluation of the growth rates showed a higher gametophyte biomass accumulation on the MFAV medium comparedto the MS½ medium (Fig. 2).

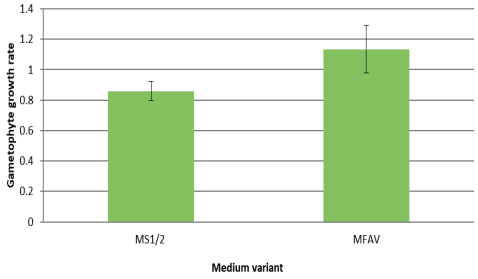

Figure 2. The growth rate of *O. regalis in vitro* gametophyte cultures

O. regalis, like most ferns, is a plant adapted to grow in poor nutrient media. Fernandez et al., 1997 and 1999 demonstrated the autotrophy of the gametophyte of these species, which can grow on sucrose-free media. The same authors observed, similar to our results, a reduction in gametophyte growth when ammonium and sucrose were added to the Knop medium (Knop, 1865).

Differentiation of sporophytes from the gametophyte culture by sexual reproduction
Sporophytes began to differentiate on gametophyte culture after three months of cultivation on the MFAV medium (Figure 3) . A layer of liquid medium applied on the gametophyte surface had a positive effect on sporophyte differentiation.
After four months of subcultivation, an exponential increase in the number of sporophytes was observed in cultures supplemented with liquid medium (Figure 4). This increase was maintained after six months, the number of sporophytes being almost double

compared to those without liquid medium applied to the gametophyte surface.

Figure 3. The sporophyte differentiated on gametophyte culture in *O. regalis*

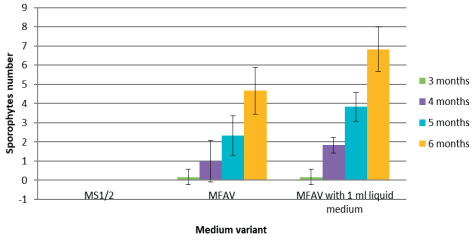

Figure 4. The number of sporophytes differentiated on different culture media

These results suggest that the presence of fluid on the gametophyte surface contributed to the fertilization process, by facilitating the mixing of gametes from antherozoids with archegonia differentiated on the gametophyte surface (Figure 5). Our cultures were unable to differentiate sporophytes on solid MS ½ medium during the cultivation period.

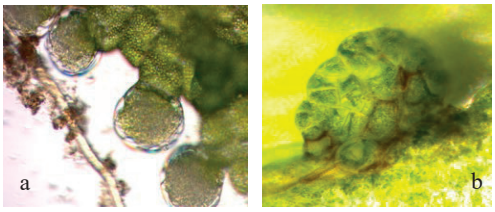

Figure 5. Antheridia (a) and archegonia (b) differentiated in gametophytes culture

However, other authors have shown that gametophytes produced archegonia and antheridia in large numbers on MS½ and MS¼ media (Markowski et al. 2016), these cultures being able to produce sporophytes after 15 months. The authors confirmed, also, sexual reproduction in this species through flow cytometry studies.

In our previous studies, cultivation on MS½ medium and the gametophyte homogenization technique by mortaring provided favorable results for other tested species such as *Polypodium vulgare*, *Asplenium trichomanes* and *Athyrium filix-femina* (Aldea, 2016), but for the species *O. regalis* this technique did not have positive results (Mitoi et al., 2024).

The formation of sporophytes on the surface of the gametophyte in *O. regalis* was described for the first time, in gametophyte cultures on Knopp medium after 6 months of cultivation at a low rate of 2.5 sporophytes/g fresh weight gametophyte (Fernandez et al., 1997). This medium does not contain NH_4NO_3, but contains 625 mg/l KNO_3. Later, the same authors reported an increase in sporophyte differentiation to approximately 20 sporophytes/g fresh weight, when the gametophyte was cultured in the absence of nutrients, on a medium containing only distilled water and 0.7% Difco Bacto-agar (Fernandez et al., 1999). Other authors obtained sporophyte differentiation in 15-20% of gametophytes on MS medium modified by replacing the macroelements with those of H&A medium (Hoagland and Aron, 1950) after three months of subcultivation (Morini, 2000). The best results were reported by Makowscki et al., (2016) on MS1/8 medium without NH_4NO_3 and without vitamins after 4 months of cultivation. These authors found, by using Knopp medium, diluted MS media, but also by eliminating ammonium and vitamins from the MS medium, that both the dilution of the culture medium and the absence of ammonium were important for achieving the fertilization process.

Other studies suggested that both the nitrogen source (NH_4NO_3 vs KNO_3) and its level may regulate sporophyte formation in sexual reproduction in other species such as *Equisetum arvense* and *Adiantum capitum-venere* (Kuriyama et al., 1992 and 2004).

All these studies showed that nitrogen, which is an important resource for heterotrophic plants, had negative effects on sporophyte formation in *O. regalis*. Not only the elimination of the

supplementary 1.600 g/l NH_4NO_3 from the composition of the MS medium had beneficial effects on the formation of sporophytes, but also the dilution of this medium, which also contains KNO_3 in a concentration of 1.900 mg/l had a beneficial effect. By diluting the MS medium to MS1/8, the remaining KNO_3 concentration was approximately 237.5 mg/L, making it the best option.

The difficulty of sporophyte formation in this species on nitrates and nitrites enriched media may also represent an explanation for its disappearance from characteristic areas. Climate change, as well as agricultural practices of large-scale use of nitrogen-based fertilizers, have probably contributed to the restriction of the spread of this species, through the impossibility of sexual sporophyte differentiation in this species at higher nitrogen concentrations.

Evaluation of sporophytes development

Four types of culture media were tested for evaluation of sporophytes roots and leaves growth. The best results were also obtained on the MFAV medium, the sporophyte growth rate being 1.65±0.25 at 3 months. The media supplemented with hormones had no beneficial effects on sporophyte development, both at the root and leaf level, except for the number of differentiated leaves in the MSR variant (Table 2). Following the measurements performed, it was observed that sporophytes can also develop on the MS½ medium, although the analysed morphometric parameters were significantly lower than MFAV, but registering higher values compared to the other experimental variants with hormones (MSR and E7).

Table 2. Gravimetrical and leaf morphometrical parameters of sporophyte development on different culture media at three months after inoculation

Nutritive medium variants	RN (cm)	LRL (cm)	SRL (cm)
MS½	1.5±1.04	0.21±0.07	0.18±0.16
MFAV	1.83±0.75	0.28±0.09	0.28±0.05
E7	0.16±0.40	0.01±0.04	0.01±0.04
MSR	0.16±0.40	0.01±0.04	0.01±0.04

These results are also supported by previous studies in *O. regalis* which showed that supplementation with hormones such as benzyl adenine (BA), gibberellic acid (GA3) and naphthaleneacetic acid (NAA) led to vitrification of sporophyte tissues (Morini, 2000). Subsequently, in this study sporophytes were grown on H&A medium without hormones which favoured vigorous development of leaves and roots, but differentiation of new leaves or roots was rarely observed. In contrast, our results showed a leaf differentiation rate of 1.5±0.83/3 months and 6.33±2.5/12 months of cultivation, while root differentiation was 1.83±0.75/3 months and 6.83±1.16/12 months of cultivation on MSFAV medium (Table 3).

Table 3. Roots morphometrical parameters of sporophyte development on different culture media at three months after inoculation

Nutritive medium variants	SGR	LN	LLL (cm)	SLL (cm)
MS½	1.04±0.21	0.83±0.40	0.31±0.13	0.35±0.25
MFAV	1.65±0.25	1.5±0.83	0.6±0.14	0.43±0.12
E7	0.11±0.10	-0.16±0.75	0.1±0.06	0.08±0.07
MSR	0.14±0.11	1.16±1.32	0.2±0.12	0.11±0.09

Multiplication by sporophyte division at the rhizome level

Our experiment showed that the development of sporophytes were not significant affected by the rhizome sectioning (Figure 6).

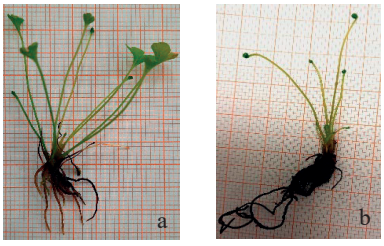

Figure 6. Undivided (a) and divided sporophyte (b) development

The growth rate reached the value of 6.25±0.47, close to that recorded for undivided sporophytes of 6.52±1.15 after one year of cultivation on MSFAV medium (Table 4). However, the number of leaves at the level of the divided sporophyte was not recovered, the difference being clearly superior in undivided sporophytes. However, at the root level, a higher root length was recorded in the divided sporophyte, but the difference was not significant.

Table 4. Comparison between gravimetrical and morphometrical parameters of divided and undivided sporophytes at twelve months of growth on MFAV medium

Variants	SGR	LN	LLL (cm)	SLL (cm)	LRL (cm)
Divided sporophytes	6.25±0.47	2.5±0.83	1.58±0.44	-0.28±1.13	2.98±1.14
Undivided sporophytes	6.52±1.15	6.33±2.5	2.28±0.92	-0.26±0.55	2.86±0.85

Obtaining gametophytes from leaves and roots fragments

After 20 days of inoculation the rootlets detached from sporophytes cultured on MFAV medium differentiated gametophytic prothalli, while leaf explants generated gametophytic-like structures after a few months (Figure 7).

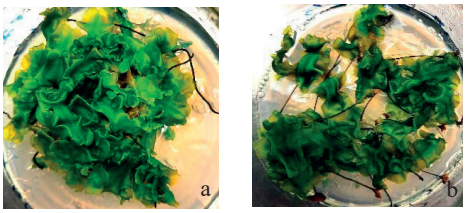

Figure 7. Aposporic gametophytes regenerated from rootlets (a) and leaflets (b)

Lawton (1936) reported the obtaining of diploid gametophytes (with antheridia and archegonia) and tetraploid sporophytes, in the species *O. regalis*, by inoculating, under sterile conditions, of explants from primary leaves of diploid sporophytes on Shieve minimal medium containing only 200 mg/l $Ca(NO_3)_2$. In contrast, the root tips did not differentiate into gametophytes.

In our experiment, the direct production of gametophytes from sporophyte tissue confirmed the occurrence of apospory. The induction of apospory demonstrates that the gametophyte can be generated from somatic tissues without the usual process of sporogenesis. The gametophyte formed by apospory retains the diploid number of chromosomes, unlike the usual gametophyte, which is haploid. In Nakamura and Maeda (1995) obtained aposporic gametophytes from fragments of excised sporophyte leaflets from the species *Lygodium japonicum* L., which were used for the isolation of protoplasts. Marimuthu et al., 2022, mention several factors that influence the induction and subsequent development of aposporic gametophytes including the detachment of organs from the mother plant, homogenization of sporophyte tissues, culture media with low or absent concentrations of sucrose.

Acclimation of sporophytes to potted soil

To ensure an efficient transition from the *in vitro* to the *ex vitro* environment, plants must progressively adapt to the low humidity, high light intensity, autotrophic nutrition and non-sterile conditions specific to greenhouses and experimental fields.

In order to obtain *O. regalis* plants, sporophytes obtained by *in vitro* culture were acclimatized to potted soil conditions. In a first experiment, three variants of both sterile and non-sterile substrate were tested. Subsequently, four more substrate variants were used to determine the adaptation of this species to different soil types. During the first month of acclimatization, a slight decrease in the weight of the sporophytes was observed, attributed to dehydration resulting from the transition to lower chamber humidity. Some leaves dried out, while the remaining ones expanded their foliar area. The acclimatization rate to *ex vitro* conditions, was 100%, after two months of potted culture, for all substrate variants except S1. This substrate proved to be unrecommended for this species, as all potted plants on this substrate did not survive (Table 5).

Table 5. The survival rate after two months in acclimatization conditions on different potted substrates

Substrate variants	% survival rate
Substrate for indoor plants S1	0
Sterile S1	0
Substrate for sowing and propagation S 2	100
Sterile S2	100
Substrate for ornamental shrubs S3	100
Sterile S3	100
Substrate for aquatic plants S4	100
Sterile S4	100
Substrate for acidophilous plants S5	100
Sterile S5	100
Substrate for cacti S6	100
Sterile S6	100
Natural peat substrate S7	100
Sterile S7	100

Moreover, other studies have also shown a 100% acclimatization of *O. regalis* plants obtained in vitro, to *ex vitro* culture conditions using sterile natural peat as a substrate with a pH of 6.5 (Makowski et al., 2016) or a 3:1 mixture of peat and perlite (Morini, 2000).

Potted plant growth

The development of potted plants under greenhouse conditions was monitored at 6 and 12 months of growth on two substrate variants: S2 containing 80% peat and 20% humus and S3 with bark enriched with nutrients and trace elements (Figure 8).

Figure 8. Potted plants at four and twelve months under greenhouse conditions

The best growth rate in terms of weight gain was observed on S2, both at 6 and 12 months (Table 6).

The variants with the autoclaved substrates recorded lower values than those in which the non-sterile substrate was used.

The survival rate of potted plants after 12 months of cultivation decreased, reaching from 83% for plants grown on substrates of variant 2 to 50% for plants grown on sterile substrate of variant 3.

Table 6. The growth and survival rate of potted plants on different substrates at 6 and 12 months in greenhouse

Substrate	RL (cm)		RV (µl)	
	6 m	12 m	6 m	12 m
S2	3.55±1.1	23.42±1.2	358.33±20.4	1260±418.3
Sterile S2	2.76±1.3	22.68±1.5	416.66±40.8	1520±670.8
S3	1.53±0.5	19.52±1.1	325±41.8	1375±150
Sterile S3	2.22±1.3	19.33±0.5	330±27.3	1366.66±57.7

The number of leaves was reduced from 8-11 leaves/sporophyte to 4-5 leaves/potted plant after one year of greenhouse culture.

The leaves undergone shape and size changes, starting to resemble the fronds from natural conditions (Figure 8).

The dimensions of the largest and smallest leaves also showed better growth on S2 substrate (Table 7).

Table 7. Morphometrical leaf parameters of potted plants on different substrates at 6 and 12 months in greenhouse

Substrate	LN		LLL (cm)		SLL (cm)	
	6 m	12 m	6 m	12 m	6 m	12 m
S2	-4±2.8	-5.2±2.7	2.86±0.4	5.76±1.4	2.9±0.5	1.2±0.8
Sterile S2	-1±1.4	-3±2.5	1.66±0.5	4.94±0.5	1.63±0.7	0.74±0.7
S3	-4±2.9	-4.5±2.8	1.12±1.7	3.72±1.8	2.55±0.9	0.04±1.1
Sterile S3	-2.8±2.5	-5.33±3.7	1.6±0.6	5.06±0.5	1.52±1.7	2.13±1.2

Compared to the vegetative system, notable development occurred at the root level between 6 and 12 months (Figure 9).

Figure 9. Development of roots system at 12 months after transfer to potted soil

The radicular volume was 4 times greater than at 6 months, while the root length reaches from 3.55±1.15 cm at 6 months to 23.48±1.28 cm at one year for substrate variant 2, which was more advantageous than the other variants (Table 8). For variant 2, the radicular system volume was higher for the sterile substrate, than the non-sterile one. For variant 3 this sterile-unsterile difference was not preserved.

Table 8. Morphometrical root parameters of potted plants on different substrates at 6 and 12 months in greenhouse

Substrate	SGR		SP (%)	
	6 m	12 m	6 m	12 m
S2	3.77±0.94	2.11±0.42	100	83
Sterile S2	2.19±1.28	1.93±0.27	100	83
S3	3.20±1.29	1.93 ±0.18	100	66.67
Sterile S3	2.61±0.78	1.99±0.13	83.33	50

CONCLUSIONS

In conclusion, the results of our study indicate that the species *O. regalis* prefers nutritive media with low nutrient concentrations, a characteristic common to the majority of fern species, having though a specific requirement for nitrogen-poor environments for both gametophyte development and sporophyte differentiation and development. The high percentage of acclimatized plants suggests that the species *O. regalis* can also adapt to other types of substrate depending on environmental conditions, offering opportunities for the restoration and conservation of ecosystems by reintroducing the species into natural environments.

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