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RESEARCH ARTICLE

STUDIES ON REGENERATION OF GAMETOPHYTES AND MASS MULTIPLICATION OF *Anemia rotundifolia* schrad. (PTERIDOPHYTE)

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ABSTRACT

Anemia rotundifolia, a rare fern species was studied to determine that can explants of gametophyte be used to multiply the plants (gametophytes and sporophytes) and also to understand the reproductive barriers. Explants were sown on Parker's & Thompson's culture media. Study demonstrated that explants produced secondary regenerates which matured into cordate gametophytes and bear only archegonia. These regenerates gave rise numerous tertiary gametophytes that bear female and male gametes intermixed showing monoecious sexuality. Secondary regenerates had option for intergametophytic selfing, as it bears only archegonia. Tertiary regenerate exhibiting female and male gametes performed both inter and intragametophytic selfing, as a result each tertiary regenerates produced multiple sporophytes. Secondary gametophytes did not produce any antheridia and sporophyte, indicating that establishment of sporophytes on tertiary regenerates has reciprocal impact on secondary gametophytes development. Study concluded that apical explants of gametophyte in *A. rotundifolia* may be used for mass multiplication of gametophytes and sporophytes, and also to ensure its in vitro conservation. Expression of archegonia on secondary regenerates and presence of both the gametes on tertiary regenerates confirmed that the moderate heterozygosity and homozygosity could be cause of reproductive barriers and success in consecutive regenerates.

INTRODUCTION

Anemia rotundifolia Schrad., native to south Brazil (Schrader, 1824), also conserved in the ferneries of Calicut University and National Botanical Research Institute, Lucknow, India respectively. It is an ornamental fern and belongs to the categories of threatened species. The ontogeny and study on reproductive behaviours of threatened species may provide an insight about cause of their threat. Thus, an attempt of spore culture and ontogeny was performed to understand the possible cause of reproductive barriers and unusual establishment of sporophytes (Srivastava *et al.*, 2009). Present study demonstrated that the gametes fusion occurs by both inter and intragametophytic selfing mechanism, but the percentage of sporophyte productivity was lesser (20%) in intragametophytic selfing than to intergametophytic selfing (75%). Findings also concluded that the gene pool of *A. rotundifolia* is moderately heterozygous and chances of colonization could be through spread of spores and intergametophytic selfing. In these circumstances it is necessary to enquire that can multiple gametophytes and sporophytes in *A. rotundifolia* be produced from explants of gametophyte? Although many studies were made to understand ontogeny, reproductive behaviour, cause of threat for survival and mass propagation of various fern species performing spore culture (Klekowski 1969*a*, *b*; Nayar and Kaur 1971; Schedlbauer and Klekowski 1972; Schedlbauer 1976; Haig and Westoby 1988; Banks 1999; Verma and Mani Selvan 2001; Ranker and Houston 2002; Huang *et al.*, 2004; Flinn 2006; Goller and Rybczynski 2007; Soare *et al.*, 2007; Khan *et al.*, 2008; Soare 2008; Hua *et al.*, 2010; Lazar *et al.*, 2010; Maridass *et al.*, 2010; Behera *et al.*, 2011; Marimuthu and Manickam 2011). But only few attempts were made to produce multiple gametophytes and sporophytes from isolate gametophyte explants. In addition, the vegetative propagation from excised leaves of *Osmunda cinnamomea* (Sussex and Steeves 1953; Steeves and Sussex 1957) and *Microgramma vacciniifolia* (Hirsch 1975), old prothalli (Mottier 1938; Nayar and Kaur 1971), rhizome meristem of *Matteuccia struthiopteris* (Hicks and Von, 1986; Thakur *et al.*, 1998), gemmation in gametophytes of *Osmunda regalis* (Fernandez *et al.*, 1997), scales in *Platycerium bifurcatum* (Dolinsek and Camloh 1997), apices and stolon of *Nephrolepis exaltata* (Lazar *et al.*, 2010) and behaviour of gametophytes (Chiou and Farrar 1997; Khare 2001) in certain fern species under prolonged culture were observed. Study on growth and developmental pattern of gametophytes and sporophytes (Nester and Coolbaugh 1986; Hickok *et al.*, 1987; Miller and Wanger 1987; Melan and Whittier 1990), and the tissue culture technique were used as a tool for plant regeneration and large scale propagation of various fern taxa (Padhya and Mehta 1982; Higuchi *et al.*, 1987). In recent early, investigations showed that culture of homogenized gametophytes and sporophytes in culture medium can contribute to increase sporophyte production in certain fern species (Fernandez *et al.*, 1999; Soare 2008; Soare *et al.*, 2010; Somer *et al.*, 2010), but efforts to propagate species using gametophyte pieces

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(explants) were least attempted. Attempt to raise multiple gametophytes and sporophytes by homogenization of gametophytes in *Woodwardia virginica*, *Dryopteris affinis*, *Osmunda regalis* and *Pteris ensiformis* were made by Fernandez *et al.*, (1999), where they produced approximately two thousand sporophytes after two months of culture. They recommended that the homogenization of gametophytes can be considered to be excellent method for propagation of species having short life cycle. Regeneration of gametophytes in *Ampelopteris prolifera*, *Pteris vittata* and *Woodwardia orientalis* determined two different mode of regeneration pattern (Khare 2001). In *A. prolifera*, regeneration begin only from apical meristem region, however in other two species it took place from the wings margin. Secondary gametangial ontogeny also differed and was two types. Regenerating archegoniate gametophyte in *A. prolifera* increased opportunity for intergametophytic mating with newly developed antheridiate gametophyte. In *P. vittata* and *W. orientalis*, the regenerating single gametophytes produced composite populations of clone having both male and female gametangia. This peculiar sequence of gametangia occurrence increases possibility of intra as well as intergametophytic selfing and crossing allowing species for colonization (Khare 2001). Green and incompletely differentiated sporangia were also utilized to initiate the in vitro culture in certain apogamous fern species. In these ferns the secondary gametophytes were formed through: (i) the branching of the prothallic filaments where each ramification represent initial stage of new prothallus, (ii) elongation of the cells in the apical area, which represent the initial cell of new prothalli, (iii) cells that also function as prothallic initials in base area, (iv) ramification of the prothalli (Soare 2008; Soare *et al.*, 2010).

But, it could not elucidate gametangial involvement for sporophyte establishment, since the taxa adopted apogamous tendency. More interrelated effort to induce sporophytes from spore derived gametophytes and the potential of homogenized sporophytes were investigated in ferns (Somer *et al.*, 2010). It was also claimed that the morphogenesis in ferns might also be induced by mechanical disruption or homogenization of gametophytes and sporophytes and subsequent culture on appropriate culture media (Fernandez *et al.*, 1993, 1997). Since, *A. rotundifolia* is conserved under captivity in Indian boundaries and also becoming threatened in its natural habitat. As such except ontogeny (Srivastava *et al.*, 2009), any attempt is not made on this particular species to understand and investigate the possible mechanism of propagation and conservation using single gametophyte as resource material. Therefore, present investigation is with an intrinsic aim to explore alternative mechanism of propagation and produce sporophytes through in vitro technique. Objective is also to evaluate the survival extant of explants, potentiality to give rise subsequent regenerates, growth rate, expression of sexual phenotypes and sporophyte production.

MATERIALS AND METHODS

Spore collection

Frond of *A. rotundifolia* was collected in November 2011 from sporophyte plants growing in the fernery of National Botanical Research Institute, Lucknow, India. Frond was dried at room temperature and left in desiccator for a week to dehisce the sporangia for release of spores.

Preparation of culture media and sterilization of spores

P&T culture media comprising macro (Parker's) and micro (Thompson's) elements was prepared in 1 litre distilled water and solidified with agar powder. Culture media was autoclaved at 121°C for 15 min to avoid contamination (Klekowski and Lloyd 1968; Klekowski 1969*a*, *b*). The pH of media was maintained at 5.8 before adding the agar 1% (w/v). Spores were surface sterilized with 2% sodium hypochlorite solution (for 2 minutes) and subsequently rinsed thrice with sterilized distilled water.

Spores inoculation and gametophyte development

Sterilized spores were inoculated on petridish containing culture media. Petridish (containing spores) was left in the culture room at 65 µmol m^{-2} sec^{-1} light intensity, photoperiod of 10:14 hours (light and dark), 23±2°C temperature for spore germination and gametophyte development.

Gametophytes and preparation of explants

Matured gametophytes were isolated from the Petridish and surface sterilized with 0.1% mercuric chloride solution (for 2 minutes) subsequently rinsed thrice with sterilized double distilled water. Apical region of gametophytes were cut in to small pieces (2-3 mm) by sterilized blade in aseptic conditions. Pieces of sterilized gametophytes were ready as explants for inoculation on media.

Inoculation of gametophyte explants

Sterilized explants (15 explants) of gametophytes were inoculated on petridisesh of 8.0 cm diameter (three replicates) containing Parker's and Thompson's culture media. Petridishes (containing explants) were incubated in the culture room at 65 µmol m^{-2} sec^{-1} light intensity, 23±2°C temperature with a photoperiod of 10:14 hours (light and dark) and left for cell induction, gametophyte regeneration, sexual expression and sporophyte production. Quantitative data on the growth and development of explants, regenerate gametophytes, sex gamete expression and sporophyte production were made counting the developmental stages (Table. 1). Morphological observations were made under Nikon eclipse 80i Microscope and the photographs were taken using a camera DS Fi 1.

RESULTS

The sterilized spores sown on P&T media, after germination and subsequent development of filamentous, spathulate, cordate gametophytes grew in to mature gametophytes on 45th day of sowing. Explants taken (fifteen) from each gametophytes (45 days old) shown tremendous ability for regeneration of gametophytes and production of sporophytes performing inter or intragametophytic selfing (Fig. 1A—1C). Explants showed variable survival potentiality with a ratio of 11/15, 9/15, 8/15 (Table 1). Inoculated explants, which initially were fluorescent green, turned to dark green repairing tissues in the excised region and forming a brown thickening on the injured region on 3rd day of inoculation. Exterior cells of thickened and multi-stratose explants became unusual comprising smaller cells. These smaller chlorophyllous cells behave as meristem and cut the cells in upward orientation. This resulted initiation of secondary gametophyte on explants.

Table 1. Gametophyte explants, survival, regenerates and sporophyte development in *A. rotundifolia*.

Gametophytes used	Gametophyte explants used	Surviving explants	Explants survival percentage	Regenerates produced	Sporophytes produced	Sporophyte in ratio to explants
1	15	11	73.33%	96	29	193.33%
1	15	9	60.00%	71	22	146.67%
1	15	8	53.33%	69	10	66.67%

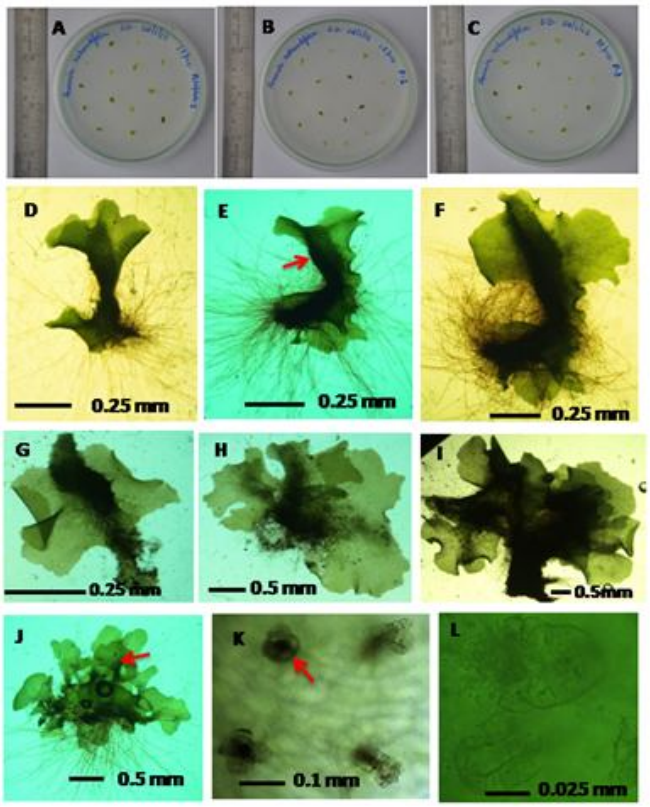

Fig. 1 Gametophyte explants culture, regenerates and female gametes expression in *A. rotundifolia.* A-C, Gametophyte explants grown on P&T culture media. D, Secondary regenerates developing on gametophyte explants. E, Secondary regenerates with thickened medial region and archegonia. F—G, Secondary regenerates with thickened medial regions and undulate dentate margins. H—I, Forked and multi-lobed secondary regenerates. J, Numerous tertiary regenerates. K, Archegonia on tertiary regenerates. L, Magnified view of archegonia on tertiary regenerates.

The cells in basal and inner layer of multi-stratose explants remained larger. Numerous hyaline initial cells emerged out of these cells, which later matured into rhizoids. Transverse and vertical division in the meristem tissues (developing in upward orientation) led to form a semi-cordate secondary gametophyte on 10th day. Numerous rhizoids also became apparent after proliferation of rhizoidal initials (Fig. 1D). Cells in the basal region of semi-cordate gametophyte thickened and became smaller leading to transform a cylindrical base. The semi-cordate secondary gametophytes develop laterally and on apex, as a result it grew into an elongated unusual gametophyte with undulate margins. Thickened cylindrical tissues became apparent up to apex of the gametophyte which borne enormous rhizoids (Fig. 1E) towards basal portion on 20th day. Simultaneously, numerous cells on this undulate elongated gametophyte also became distinct which later matured in to the archegonia (Fig. 1E arrow) on 25th day. None of the secondary gametophytes could produce antheridia during same period of development or thereafter. Wings of the gametophyte expand laterally with 2-3 celled long denticulations, whereas the thickened cylindrical tissues continuously proliferate in upward direction (Fig. 1E-G). The unusual gametophyte with undulate margin forked irregularly forming 2-3 lobed gametophytes on 30th day (Fig. 1H). The medial cylindrical tissues thickened and became brown showing degeneration of cells (Fig. 1I). As a result explants in all petridishes shown variable survival potentiality (Table-1). On 40-45th days, numerous regions on unusual gametophytes performed the meristematic activity. Cells in these meristematic regions became smaller and chlorophyllous. Meristematic region cut the cells tangentially and transversely, which led to shuttle the vegetative tissues obliquely widening and thickening the emerging regenerates. Meristematic regions apparently seem to be comprised of multi-stratose layer. The basal cells in multi-stratose layer connects with the secondary gametophyte, however apical cell by efficient cell division elongate to form regenerates on secondary gametophyte. This resulted a number of tertiary regenerate gametophytes on secondary gametophyte (Fig. 1J arrow). Each secondary gametophyte bears regenerate gametophytes on average up to 8-9 folds (Fig. 1J). The regenerates were fluorescent green. Regenerates exhibited distinct cells at the medial and apical region. The distinct cells near medial region divided transversely and vertically (as in the secondary gametophyte of explants origin), which later turned and developed into archegonia on 60th day (Fig. 1K—1L). In addition to these distinct cells in apical and marginal region, certain cells in medial region (near to archegonia) became globose and elevate out of the vegetative cells, which

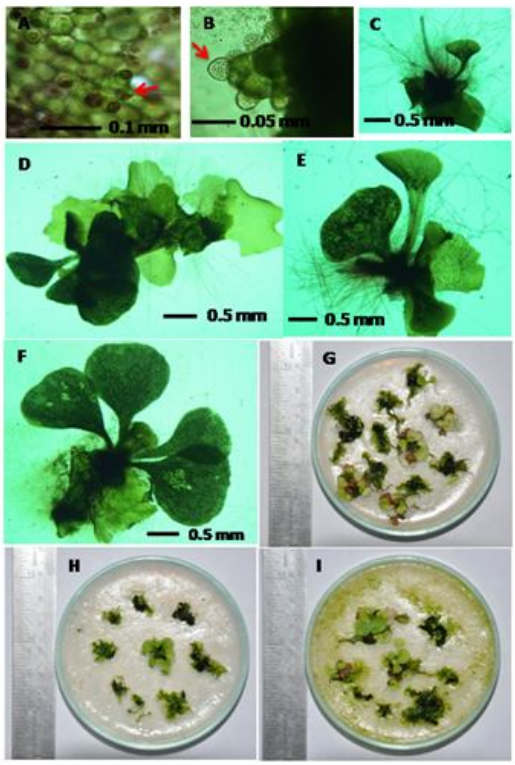

Fig.2. Expression of male gametes on tertiary regenerates and development of sporophytes in *A. rotundifolia.* A, Antheridia on tertiary regenerate gametophyte. B, Magnified view of antheridia on tertiary regenerate. C—F, Developmental stages of sporophytes. G—I, Surviving gametophyte explants and sporophyte success.

later matured in to antheridia (Fig. 2A—2B arrow). Protoplasmic content of these globose cells were granulated and highly chlorophyllous, which later developed into flagellate antherozoids in the antheridia on 70th day. Thus, male and female gametangial initials were intermixed with an overlap expression. Presence of both the female and male gametes on tertiary regenerate gametophyte had shown its monoecious tendency with an overlap expression of sex gamete. Female and male gametes were produced on same (with a short overlap expression) and different (with same stage and age) tertiary regenerate of common parental secondary gametophyte origin. Both the gametes from individual or different regenerates were available for fusion (intra or intergametophytic selfing). The female and male gamete fused to perform fertilization and production of sporophytes (Fig. 2C—2F). Single gametophyte produced up to 96 regenerate gametophytes, 29 sporophytes (Table-1) showing complete success of gamete fusion through intra or intergametic fusion (Fig. 2G—2I). Apical explants from single gametophyte produced multiple gametophytes (nine fold) and sporophytes (193.33%) in *A. rotundifolia* (Table 1). It was observed that the secondary gametophyte borne only archegonia (Fig. 1E), but not the antheridia, showing lethality effect in the first clone gametophyte. This is presumed here as it could be the impact of moderate heterozygosity in parent explants gametophyte. Both the gametes expressed in tertiary regenerates, where all the regenerates were third generation and clone of secondary gametophytes comprised of homozygosity, atleast more than the parent explants and secondary gametophytes. Thus, the homozygosity could accelerate expression of both the gametes performing intra and intergametophytic selfing. Observation asserted that since these regenerates were produced from the common parent isolate secondary gametophyte (homozygous), thus they remain obligate homozygous. The homozygosity of isolate gametophyte was carried forward to the regenerates, which compassionate for the intragametophytic selfing and intergametophytic selfing. Thus, the sporophytes were produced by the gametic fusion from same or different regenerates of common parent secondary gametophyte which further determines the intra and intergametophytic selfing. Result demonstrated that explants of individual gametophyte produced from single spore can produce multiple gametophytes (up to 96) and sporophytes (29) showing potentiality for large scale multiplication of gametophytes and sporophytes in *A. rotundifolia.*

DISCUSSION

A. rotundifolia, a threatened fern species was studied to understand its ontogeny. Mode of reproduction was observed by both inter and intra gametophytic selfing, but the percentage of sporophyte production through intragametophytic selfing were less than to intergametophytic selfing. It was perceived that the gene pool was moderately heterozygous and chances of colonization and spread through spores could be in the area of its occurrence only (Srivastava *et al.,* 2009). In view of underperformance to develop multiple sporophytes, it was thought that can apical explants of gametophytes could serve as alternative way to produce multiple gametophytes and sporophytes. Thus, a subsequent approach for explants culture was performed. Gametophyte explants culture revealed that secondary gametophytes arise from the medial or excised region of explants, but never from the apical or intact margin region, as was observed in the extended gametophyte culture of *Ampelopteris prolifera*, *Pteris vittata* and *Woodwardia orientalis* (Khare 2001). In *A. prolifera,* regeneration begins only from apical meristem region however in other two species it took place from the wings margin. In *A. rotundifolia* the secondary regenerates exhibit only archegonia not the antheridia, showing functional dioecious tendency (during observation) confirming the intergametophytic selfing to be a possible mode of reproduction.

The tertiary regenerate gametophytes exhibit both the gametangia (female and male) with an overlap expression. This made gametes available on secondary (archegonia) and tertiary regenerates (archegonia and antheridia) respectively for inter and intragametophytic selfing, supporting to the observations on *A. prolifera, P. vittata* and *W. orientalis* (Khare 2001). He noticed that the regenerating archegoniate gametophyte in *A. prolifera* increased opportunity for intergametophytic mating with newly developed antheridiate gametophyte, as also observed in *A. rotundifolia*. Regenerating single gametophytes produced composite populations of clone having both male and female gametangia in *P. vittata* and *W. orientalis* (Khare 2001). Similarly, in *A. rotundifolia* the secondary gametophytes produced multiple regenerates where all of them borne both the female and male gametangia with an overlap expression. Presence of gametes on secondary regenerates (archegonia) and tertiary regenerates (archegonia and antheridia) increases possibility of intra as well as intergametophytic selfing. Explants originated secondary gametophytes and multiple tertiary regenerates bearing both

the female and male gametes promotes high possibilities of inter and intragametophytic selfing than to the process involved with an individual gametophyte. Though on average single gametophyte usually produces one sporophyte (Sakamaki and Ino 2007), but in present attempt single gametophyte could produce 29 sporophytes confirming potentiality of explants to produce sporophytes up to 193.33% (Table 1). In addition, the survival percentage of explants were 53.33—73.33%, where as the number of regenerates produced were 69—96, out of 15 explants used, showing a high efficiency (640%) of gametophytes production. Usually single spore after germination develops into gametophyte, which by gametangia expression and other developmental progression produces one sporophyte. However, through approaches of explants culture, single gametophyte originated from one spore can produce up to 96 gametophytes and on average 29 sporophytes. Attempt of explants culture ensured multiplication of gametophytes and sporophytes at large scale. Explants culture confirmed that secondary regenerate gametophyte (borne only archegonia) laid option of gamete fusion with other secondary regenerate gametophytes (bearing antheridia). This supported to the mechanism of intergametophytic selfing.

Since the secondary gametophyte bear only archegonia not the antheridia that showed lethality effect in the first clone gametophyte which could be the impact of moderate heterozygosity in explants gametophyte. The tertiary regenerates bear both the gametes (female and male) with overlap expression laid option for both inter and intragametophytic selfing within the tertiary regenerates or the intergametophytic selfing between secondary and tertiary regenerate gametophytes. This could have possible because all the regenerates were third generation and comprised of homozygosity (at least more than the parent explants and secondary gametophytes). Thus, the homozygosity could have accelerated expression of both the gametes to perform intra and intergametophytic selfing. Since regenerates were produced from the common secondary gametophyte (homozygous), thus all of them remain obligate homozygous (at least more than to parents). This homozygosity was carried forward to the tertiary regenerates, which favoured the intra and intergametophytic selfing amongst tertiary regenerate gametophytes. Although *A. rotundifolia* naturally produces meagre gametophytes and scanty sporophytes through inter and intragametophytic selfing. But present investigation confirmed that explants culture is decisive option which can produce multiple gametophytes and sporophyte in *A. rotundifolia* from an individual spore.

Conclusion

Explants culture of isolate gametophyte produced multiple regenerate gametophytes and sporophytes ensuring its in vitro conservation. Secondary gametophytes bear archegonia but could not produce antheridia, thus they did not participate in gametes fusion. This showed the impact of lethality in secondary regenerates which had occur for the reason of moderate heterozygosity in parent gametophytes. Tertiary gametophytes bear both female and male sex gametes and performed intra as well as intergametophytic selfing, as a result multiple sporophytes were produced. This became possible because all the tertiary regenerates were third generation clone and homozygous, at least more than to parent and secondary gametophytes. This homozygosity in tertiary regenerate accelerated expression of both the gametangia to perform intra and intergametophytic selfing. Study concluded that apical explants of individual gametophyte can produce multiple gametophytes and sporophytes ensuring mass propagation and conservation of threatened species.

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REFERENCES

Banks, J.A. 1999. Gametophyte development in ferns. Ann. Rev. Plant Physiol. Plant Mol. Biol., 50:163-86.

Behera, S.K., Rawat, V.K., Singh, A.P. and Khare, P.B. 2011. Studies on the spore germination, developmental pattern and sexuality of gametophytes in *Dipteris wallichii* (R. Br. ex Hook. et Grev.) T. Moore. Indian Fern Journal, 28:172-178.

Chiou, W.L. and Farrar, D.R. 1997. Comparative gametophyte morphology of selected species of the family Polypodiaceae. American Fern Journal, 87:77-86.

Dolinsek, J.A. and Camloh, M. 1997. Gametophytic and Sporophytic Regeneration from Bud Scales of the Fern *Platycerium bifurcatum* (Cav.) C.Chr. In Vitro. Annals of Botany, 80:23-28.

Fernandez, H., Bertrand, A.M. and Sanchez-Tames, R. 1993. In vitro regeneration of *Asplenium nidus* L. from gametophytic and sporophytic tissue. Sci. Hortic., 56:71-77.

Fernandez, H., Bertrand, A.M. and Sanchez-Tames, R. 1997. Plantlet regeneration in *Asplenium nidus* L. and *Pteris ensiformis* L. by homogenization of BA treated rhizomes. Sci. Hortic., 68:243-247.

Fernandez, H., Bertrand, A.M. and Sanchez-Tames, R. 1999. Biological and nutritional aspects involved in fern multiplication. Plant Cell Tissue Organ Culture, 56:211-214.

Flinn, K. M. 2006. Reproductive biology of three fern species may contribute to differential colonization success in past agricultural forests. Am. J. Bot., 93(9):1289-1294.

Goller, K. and Rybczynski, J. 2007. Gametophyte and sporophyte of Tree Ferns In Vitro culture. Acta Societatis Botanicorum Poloniae, 76 (3):193-199.

Haig, D. and Westoby, M. 1988. Sex expression in homosporous ferns: An evolutionary perspective. Evolutionary Trends in Plants, 2:111-119.

Hickok, L.G., Warne, T.R. and Slocum, M.K. 1987. *Ceratopteris richardii*: Applications for experimental plant biology. Am. J. Bot., 59:458-465.

Hicks, G. and Von, A.P. 1986. A tissue culture of the ostrich fern *Matteuccia struthiopteris* L. Todaro. Plant Cell Tissue Organ Culture, 5:199-204.

Higuchi, H., Amaki, W. and Suzuki, S. 1987. In vitro propogation of *Nephrolepis cordifolia* Presel. Sci. Hortic., 32:105-113.

Hirsch, A. M. 1975. The effect of sucrose on the differentiation of excised fern leaf tissue into either gametophytes or sporophytes. Plant Physiology, 56:390-393.

Hua, W., Li-ping, Y., Yang, W. and Long-qing, C. 2010. Studies on in vitro culture of *Adiantum flabellulatum* from spores. Acta Horticulturae Sinica, 37(3):457-464.

Huang, Y.M., Chou, H.M. and Chiou, W.L. 2004. Density affects gametophyte growth and sexual expression of *Osmunda cinnamomea* (Osmundaceae: Pteridophyta). Annals of Botany, 94:229-232.

Khan,S., Raziq, M. and Kayani, H.A. 2008. In vitro propagation of Bird's nest fern (*Asplenium nidus*) from spore. Pak. J. Bot., 40(1):91-97.

Khare, P.B. 2001. Regeneration of Gametophytes in some homosporous ferns. Indian Fern Journal, 18:11-114.

Klekowski, E.J. 1969a. Reproductive biology of the Pteridophyta II. Theoretical considerations. Bot. J. Linn. Soc., 62:347-359.

Klekowski, E.J. 1969b. Reproductive biology of the Pteridophyta III. A study of Blechnaceae. Bot. J. Linn. Soc., 62:361-377.

Klekowski, E.J. and Llyod, R.M. 1968. Reproductive biology of the Pteridophyta I, General considerations and a study of *Onoclea sensibilis* L. J. Linn. Soc. (Bot), 60:315-324.

Lazar, A., Cerasela, P. and Sorina, P. 2010. Studies concerning the "in vitro" multiplication of *Nephrolepis exaltata* 'Fluffy Ruffles'. Journal of Horticulture, Forestry and Biotechnology, 14(3):191-193.

Maridass, M., Mahesh, R., Raju, G. and Muthuchelian, K. 2010. Clonal propagation of *Adiantum capillus-veneris*. International Journal of Biological Technology, 1(1):33-37.

Marimuthu, J. and Manickam, V.S. 2011. Ex situ conservation of two threatened ferns of the Western Ghats through in vitro spore culture. Journal of Threatened Taxa, 3(7):1919-1928.

Melan, M.A. and Whittier, D.P. 1990. Effects of inorganic nitrogen sources on spore germination and gametophyte growth in *Botrichium dissectum*. Plant Cell Envir., 13:477-482.

Miller, J.H. and Wanger, P.M. 1987. Co-requirement of calcium and potassium in the germination of spores of the fern *Onoclea sensibilis*. Am. J. Bot., 45:589-597.

Mottier, D.M. 1938. Behaviour of certain fern prothalli under prolonged cultivation. Bot. Gaz., 83:244-266.

Nayar, B.K. and Kaur, S. 1971. Gametophytes of homosporous ferns. Bot. Rev. (London), 37:295-396.

Nester, J.E. and Coolbaugh, R.C. 1986. Factors influencing spore germination and early gametophyte development in *Anemia maxicana* and *Anemia phyllitidis*. Plant Physiology, 82:230-235.

Padhya, M.A. and Mehta, A.R. 1982. Propagation of fern (*Nephrolepis*) through tissue culture. Plant Cell Reports, 1:261-263.

Ranker, T.A. and Houston, H.A. 2002. Is gametophyte sexuality in the laboratory a good predictor of sexuality in nature? American Fern Journal, 92:112-118.

Sakamaki, Y. and Ino, Y. 2007. Gametophyte contribution to sporophyte growth on the basis of carbon gain in the fern *Thelypteris palustris*: effect of gametophyte organic-matter production on sporophytes. J. Plant Res., 120:301–308.

Schedlbauer, M.D. 1976. Fern gametophyte development: Controls of dimorphism in *Ceratopteris thalictroides*. Am. J. Bot., 63:1080-1087.

Schedlbauer, M.D. and Klekowski, E.J. 1972. Antheridiogen activity in the fern *Ceratopteris thalictroides* (L.) Brogn. Bot. J. Linn. Soc., 65:399-413.

Schrader, H.A. 1824. Gottingische gelehrte Anzeigen unter der Augsicht der Konigl. 865.

Soare, L.C. 2008. In vitro Development of Gametophyte and Sporophyte in Several Fern Species. Nat. Bot. Hort. Agrobot. Cluj, 36(1):13-19.

Soare, L.C., Visoiu, E. and Andrei, M. 2007. Researches concerning the in-vitro differentiation of the fern *Phegopteris connectilis* (Michx.) Watt. Nat. Bot. Hort. Agrobot. Cluj, 35(1):7-14.

Soare, L.C., Visoiu, E. and Dobrescu, C.M. 2010. In vitro culture and regeneration of the fern *Dryopteris affinis*, species growing in a protected area. Romanian Biotechnological Letters, 50 (1):45-54.

Somer, M., Arbesu, R., Menendez, V., Revilla, M.A. and Fernandez, H. 2010. Sporophyte induction studies in ferns in vitro. Euphytica, 171:203-210.

Srivastava, R., Mishra, S. and Khare, P.B. 2009. In vitro studies of an ornamental fern: *Anemia rotundifolia* Schrad. Phytomorpholgy, 59(3-4):92-95.

Steeves, T.A. and Sussex, I.M. 1957. Studies on the development of excised leaves in sterile culture. Am. J. Bot., 44:665-673.

Sussex, I.M. and Steeves, T.A. 1953. Growth of excised leaves in sterile culture. Nature, 172:624.

Thakur, R.C., Hosoi, Y. and Ishii, K. 1998. Rapid in vitro propagation of *Matteuccia struthiopteris* L. Todaro - An edible fern. Plant Cell Reports, 18:203-208.

Verma, S.C. and Mani Selvan, P. 2001. Intraspecific variation in spore size of homosporous ferns and its implications on fern mating systems. Bionature, 21:1-9.

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