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In vitro micropropagation of the aquatic fern *Marsilea quadrifolia* L. and genetic stability assessment by RAPD markers

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Abstract
In order to conserve and multiply the aquatic fern *Marsilea quadrifolia* L., in a long-term *in vitro* procedure, the effects of different cytokinins, i.e., 6-benzylaminopurine, zeatine riboside, and N^6-(2-isopentenyl)adenine, were investigated, varying their concentration and period of supplementation. No clear stimulatory effect on the *de novo* nodes produced per explant was detected when compared with hormone-free (HF) condition. On the contrary, the rhizome explant micropropagation was inhibited, the inhibition decreasing with the decreasing strength of cytokinins, though without reaching any significant enhancement. Since, as a consequence of the tissue culture procedure, the occurrence of somaclonal variation may introduce genomic alterations, genetic stability was assessed by random amplified polymorphic DNA (RAPD) analysis by comparing eight randomly selected micropropagated plants derived from repeated subcultures, with donor plant. Eighteen different primers generated 189 bands ranging from 100 to 3250 bp, and the same banding profiles were exhibited. No genomic alterations were evidenced in any of the micropropagated plants. Well-developed micropropagated plants were also successfully acclimatized under greenhouse condition. These positive results suggest that the *in vitro* HF micropropagation could be useful in the development of *ex situ* conservation programs of *M. quadrifolia*, even in order to possibly reintroduce the plants in their natural environment.

Keywords: *Conservation, cytokinins, genetic stability,* in vitro *micropropagation, nodal explants*

Introduction

The genus *Marsilea* with its nearly 45 species is a worldwide diffuse and is found in undisturbed conditions, which can efficiently grow through a rapid vegetative reproduction, becoming even an invasive species as occurred with the allochthonous *M. azorica* Launert & Paiva (Launert & Paiva 1983) in southern USA (Schaefer et al. 2011). The species *M. quadrifolia* L. has a circumpolar distribution, inhabiting the aquatic and semi-aquatic environment, as floodplain areas. Once widely spread in Central and Eastern Europe, it has been included in the Red Lists and in the Red Data Books, belonging to vulnerable (VU) category (Lucas & Walters 1976). In fact, due to the extended drainage carried out to yield new land for crops and to the extensive channelization of rivers, its habitat has been destroyed, as it has become extinct in some areas, or deeply modified, as the few population still existing have decreased with less number of individuals (Jalas & Suominen 1972; Husák & Otahelová 1986; Käsermann & Moser 1999). Despite being now indicated as "Least Concern" in the last IUCN Red List (Gupta 2011), due to its diffusion throughout much of Southern Europe and some Asiatic countries, locally *M. quadrifolia* remains extremely rare, mainly due to the loss of adequate habitats. In Italy, the presence of *Marsilea*, represented by *M. quadrifolia* and *M. strigosa* Willd., is rather reduced and both species have been included into the international rules as The Berne Convention (1979) and The Habitat 92/43 CEE directive (1992), belonging once more to the VU category. Even if new scattered and/or occasional growing sites of *M. strigosa* (Ernandes & Marchiori 2012) and of *M. quadrifolia* have been reported (Pistoja et al. 2003), it should be remembered that their presence is, however, extremely variable year by year according to the water level fluctuations, the

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overgrowth of other aquatic species, and the modern farming practices involving the massive use of herbicides. Thus, as habitat degradation makes it difficult to develop *in situ* conservation programs, *in vitro* micropropagation from preformed meristems could be an efficient procedure for *ex situ* germplasm multiplication and conservation. This technology gives the opportunity to produce a great number of pathogen-free individuals starting from small explants, to control light and temperature, to exclude seasonal changes, and to adjust the supplementation of nutrients and growth regulators (George 1993). Notwithstanding the numerous advantages and the clear expectation of the production of genetically uniform plants, somaclonal variation, i.e., uncontrolled and spontaneous genotypic variation originating during cell and tissue culture, may occur (Larkin & Scowcroft 1981). Despite the huge amount of research to overcome it, this undesired phenomenon has been poorly understood and still remains one of the major problems of *in vitro* clonal micropropagation. This is probably partly due to *in vitro*-induced stress and can be affected by many factors, such as the type of tissue, the type of growth regulators, and the number and duration of subcultures, which determine the onset and the frequency of variation (Pierik 1987; Brar & Jain 1998), but its molecular basis is not precisely known. Although the occurrence of mutations due to somaclonal variation has been frequently used for the selection of clones that can be valuable on an agricultural and a farming point of view (Bairu et al. 2011), when the final aim of *in vitro* micropropagation is the *ex situ* conservation program of threatened or endangered species, early detection of somaclonal variants is essential since genetic instability is undesirable. Thus, genetic fidelity of the micropropagated plants must be necessarily verified. Among the molecular tools available for DNA analysis, random amplified polymorphic DNA (RAPD)–PCR analysis proved to generate reliable and affordable DNA marker for the assessment of genetic stability in micropropagated plant ranging from economically valuable clones for timber industry (Rani et al. 1995; Valladares et al. 2006) to medicinal or aromatic (Panda et al. 2007; Srivastava et al. 2009; Chuang et al. 2010; Paul et al. 2010; Saha et al. 2012) and endangered plant species (Guo et al. 2006; Mallón et al. 2010). As already aptly discussed by Mallón and coworkers (2010), even though other molecular markers are more sophisticated and reproducible, no technique can completely guarantee the genetic fidelity of the micropropagated plants. Nevertheless, RAPD–PCR ensures a good compromise in terms of (i) low operative cost (quite limiting point when applied to non-economically important species), (ii) low time-consumption, (iii) no requirement of prior knowledge of the genome to be evaluated, and (iv) coverage of a high DNA extension by fast scanning of the whole genome (Welsh & McClelland 1990; Williams et al. 1990). Thus, RAPD analysis offers a useful tool for the rapid assessment of somaclonal-induced variability in *in vitro*-micropropagated plants.

With regard to *in vitro* culture of *M. quadrifolia*, it has been previously reported that the supplementation of abscisic acid to aseptically cultured rhizome segments induces the development of land-form characteristics, as elongation of petioles and roots and increase in the surface area of leaflets (Liu 1984; Lin & Yang 1999), whereas the supplementation of auxins has no ameliorative effects on the regenerative response (Banciu et al. 2009). To the best of our knowledge, no data are available about cytokinin effect on *M. quadrifolia*, albeit some papers report the use of cytokinins on *in vitro* culture of other Pteridophytes (Morini 2000; Fernández & Revilla 2003, and references herein; Menéndez et al. 2009). Cytokinins regulate a considerable number of different developmental and physiological processes in aerial and subterranean organs, including cell division in the shoot and root meristems, chloroplast differentiation, leaf senescence, stress responses, and pathogen resistance. They have been proposed as long-range signaling molecules, but since they promote the outgrowth of axillary buds antagonizing the activity of auxin, they may also be considered as local signal for plant development (Mok 1994; Mok & Mok 2001; Werner & Schmülling 2009, and references herein). The purpose of this study was to investigate whether cytokinins play a stimulatory role on *in vitro* micropropagation of this fern, starting from *in vivo* collected sporocarps. Thus, different concentrations of 6-benzylaminopurine (BAP), zeatine riboside (ZR), or N^6-(2-isopentenyl)adenine (2iP) were supplemented to the multiplication medium for different periods and the multiplication rate obtained (i.e., the *de novo* mean node number produced per explant) was compared with that of hormone-free (HF) condition. To assess the genetic stability following on *in vitro* culture, the gene pool fidelity of the clonal population obtained by the multiplication protocol was verified by RAPD analysis, in the event of a possible reintroduction in its natural environment.

Materials and methods

Plant growth regulator solutions

N^6-(2-Isopentenyl)adenine (Sigma Chemical, St Louis, MO, USA) and BAP (Sigma Chemical) were dissolved in distilled water to obtain 3.2×10^{-2} M stock solutions that were sterilized using

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0.2 μm sterile disposable filter units. ZR (Duchefa Biochimie, Haarlem, the Netherlands) was dissolved in 70% ethanol to obtain 4×10^{-3} M stock solution.

Plant material and culture conditions

Sporocarps of *M. quadrifolia* from a plant cultured *ex situ* at the Botanical Garden of the University of Parma (Italy) were surface sterilized in the following consecutive steps: 3.3 g/l Ziram for 30 min, followed by 70% ethanol for 15 min, followed by 0.01% NaOCl plus 0.01% benzalkonium chloride for 15 min; then they were rinsed three times in sterile distilled water. Halves cut from each sporocarp were placed in Petri dishes containing $\frac{1}{2}$ strength Murashige and Skoog (MS) mineral salts and vitamins (Murashige & Skoog 1962), supplemented with 1.5% sucrose, 0.7% agar, pH 5.8 ($\frac{1}{2}$ MS medium). Cultures were incubated in the darkness at 25 ± 1°C for the first 5 days, then they were transferred at light intensity of 27 $\mu mol\, m^{-2}\, s^{-1}$ under 16-h photoperiod at the same temperature. After 6 weeks, one sporophyte among those formed from fertilized eggs was cut into three-node explants, which afterwards were cultured on $\frac{1}{2}$ MS medium in the same culture conditions described above to initiate the micropropagation program. Individual plantlets were used as donor of explants either for further micropropagation on $\frac{1}{2}$ MS medium or for multiplication experiments in the presence of different cytokinins (see below).

Multiplication protocol optimization

Groups of 4 three-node explants, obtained from several subcultures on HF medium, were cultured in Petri dishes containing $\frac{1}{2}$ MS medium in the presence of three different cytokinins, i.e., BAP, ZR, or 2iP, each supplemented at three different concentrations, i.e., 0.1, 1, or 10 μM, for three different periods of supplementations, i.e., 1, 2, or 3 weeks, respectively. The explants subjected to shorter cytokinin periods of supplementation were transferred on $\frac{1}{2}$ MS medium devoid of any plant growth regulator (HF medium) after 1 or 2 weeks, respectively, to conclude the period of 3-week *in vitro* culture. In each experiment, control was performed culturing the same type of explants for 3 weeks on HF medium. All the explants were incubated at 25 ± 1°C, at light intensity of 27 $\mu mol\, m^{-2}\, s^{-1}$ under 16-h photoperiod; after a 3-week total period of culture, the number of the *de novo* produced nodes was counted and the mean node number produced per explant was calculated. The experiments were carried out in duplicate and repeated three times.

Statistical analysis

Micropropagation experiments were conducted in a randomized design, by which the effects of different cytokinins, concentrations, and supplementation period were compared with that of HF condition, as control. Data were subjected to Levene test to check the homogeneity of variances, and were then analyzed with one-way ANOVA followed by comparison of multiple treatment with the control applying *post hoc* Dunnet test at $p < 0.01$ using SPSS 18.

Ex vitro *transplantation and acclimatization*

Thirty well-developed multiple node rhizomes micropropagated in the presence of 1 μM 2iP for 1 week, as well as 31 rhizomes obtained on HF medium, were gently taken out from culture plates, thoroughly washed with sterile distilled water to remove traces of agarized medium, and transferred to peat pots containing a sterile mixture of soil and vermiculite (1:1). Each pot was put into a glass jar full of sterile distilled water, and the jars were put in a plastic box covered with a transparent polythene film to insure high humidity; the plastic box was kept under greenhouse conditions. At the end of the first week of transplantation, the polythene film began to be partially opened until it was completely removed at the end of the third week of transplantation. Afterwards, *ex vitro* well-developed plants were used for molecular analysis.

DNA extraction and RAPD analysis

DNA was extracted from fresh expanded leaves of the donor plant and of the micropropagated plantlets. Four micropropagated plants derived from explants cultured on HF medium and four micropropagated plants derived from explants cultured on medium supplemented with 1 μM 2iP for 1 week were analyzed. DNA was also extracted from the leaves of individuals of *M. quadrifolia* collected from different northern Italy areas, namely one found in the field at S. Nazaro-Parma, the second and the third found from Colli Euganei and a site nearby Modena, which were kindly provided by the Padua Botanical Garden and by the garden center of Michele Morisi (S. Giovanni in Persiceto-Modena), respectively. Fresh material was freeze dried in liquid nitrogen, mortar grinded, and extracted according to Marieschi et al. (2011) with slight modifications: initial incubation in cetyl trimethyl ammonium bromide buffer was done at 65°C for 30 min instead of incubating overnight at room temperature and with the exclusion of activated charcoal. DNA concentration and purity

($A_{260/280}$ and $A_{260/230}$) were evaluated by spectrophotometric analysis. The suitability of DNA for RAPD analysis was also checked by ethidium bromide-stained agarose/Tris acetate EDTA (TAE) gels which allowed both to evaluate DNA integrity and to further confirm DNA quantitation by visual comparison with DNA standards. DNA concentration of each sample was adjusted to approximately 20 ng/μl.

Agarose gels were analyzed and quantitated with a Kodak DC40 camera (Eastman Kodak Company, Rochester, NY, USA) using the Kodak digital science 1D Image analysis software (Eastman Kodak Company).

PCR were performed on approximately 40 ng of DNA template with a 25-μl volume containing 67 mM Tris–HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2 mM $MgCl_2$, 0.2 mM dNTPs, 1.25 U SubTherm Taq DNA polymerase (Fisher Molecular Biology, Trevose, PA, USA), and 25 pmol specific primers. Amplification was done as follows: 94°C for 5 min, 40 cycles of 94°C for 40 s, 36°C for 40 s, 72°C for 2 min, followed by 1 cycle of 72°C for 10 min (PTC-100; MJ Research Inc., Waltham, MA, USA). A total of 20 random primers were utilized for RAPD analysis, out of which 18 random decamer primers were selected on the basis of the clarity of banding patterns. The primers selected for the analyses were OPA01, OPA03, OPA04, OPA05, OPA07 OPA09, OPA10, OPA11, OPA12, OPA20, OPB02, OPB08, OPB10, OPB12, OPB16, OPB19, OPB20, and OPP10 (Operon Technologies®, Alameda, CA, USA).

PCR products were loaded on 2% agarose gel for electrophoresis in 1 × TAE buffer. Agarose gels were analyzed and quantitated with a Kodak DC40 camera (Kodak) as stated earlier. The size of the amplicons was determined by comparison with size standard GenRuler™ 100 bp DNA Ladder Plus (MBI Fermentas, Vilnius, Lithuania). RAPD–PCR with each primer was repeated at least twice, and only repetitive bands were taken into account for the analysis.

Results

Multiplication protocol optimization

M. quadrifolia was micropropagated by culturing groups of 4 three-node explants (Figure 1a) in the presence of different cytokinins, supplemented at different concentrations, for different periods. The effects of these culture conditions were assessed as the mean node number produced per explant branching from the pre-existing nodes at the end of the 3-week period of *in vitro* culture.

Cytokinin supplementation did not significantly enhance the number of nodes produced per explant in comparison with that of the control condition (7.1–7.9 mean node number according to the set of experiments); on the contrary, an increasing significant inhibitory effect was observed due to both the cytokinin strength and the supplementation period length (see Figure 2).

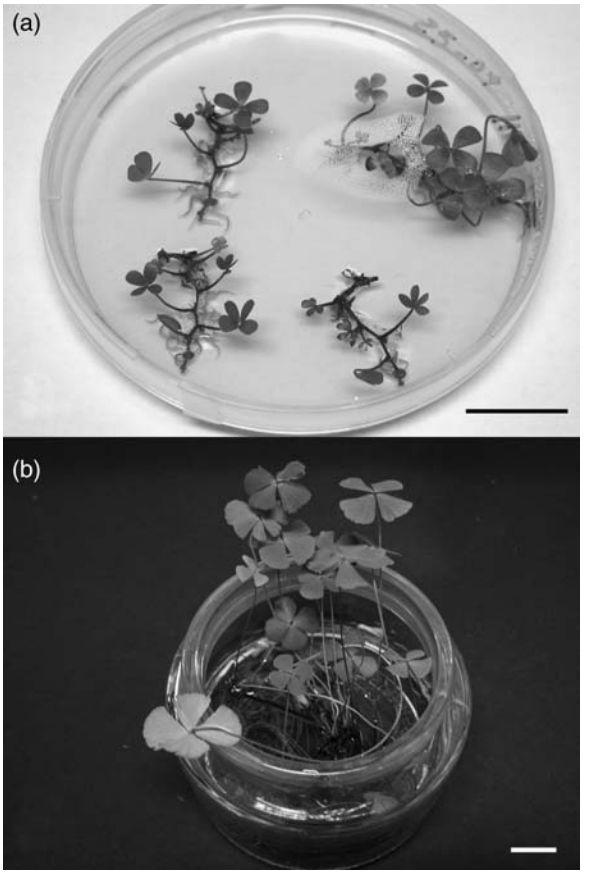

Figure 1. Different stages of *ex situ* multiplication protocol of *M. quadrifolia*. (a) Example of 4 three-node explants *in vitro* cultured. (b) Three-month acclimatized plant. Scale bar: 2 cm in both.

In fact, micropropagation was always negatively affected by BAP at each tested concentration with the inhibition being as deep as longer was the period of supplementation. After the shortest exposure at the lowest concentration (1 week, 0.1 μM), indeed the mean node number produced was more than halved than that of the control. Complete inhibition, with less than one *de novo* node produced, was reached after 3 weeks at the highest concentration.

ZR supplementation at the highest concentration (10 μM) resulted in significant inhibitory effect regardless of the length of the treatment, whereas at the lower concentrations (0.1 and 1 μM), inhibition became significant only after 3 weeks of supplementation.

No inhibitory effect was observed when the explants were cultured in the presence of 0.1 or 1 μM 2iP for 1, 2, or 3 weeks; a very slight increase in the mean node number was detected after the sup-

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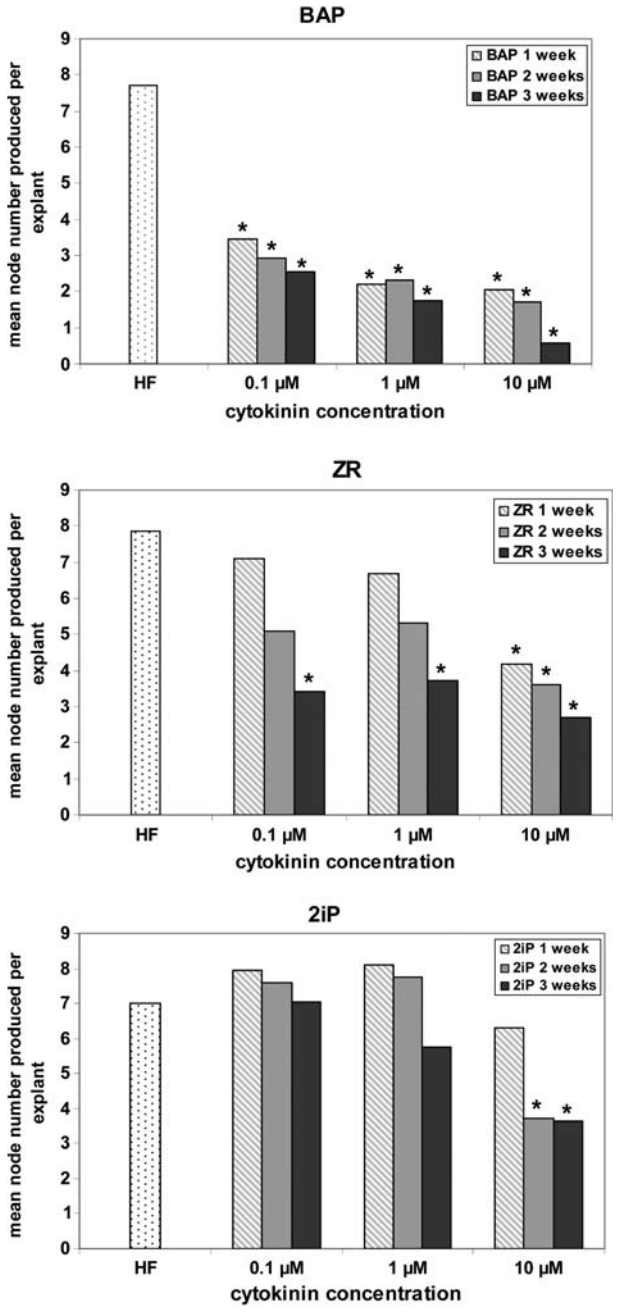

Figure 2. Mean node number produced after culture in the presence of different cytokinins. Each cytokinin was supplied at three different concentrations for 1, 2, or 3 weeks. At the end of the shorter cytokinin exposures, explants were transferred on to HF medium till the end of the culture. Counting was done at the end of 3-week culture. HF: culture on hormone-free medium for 3 weeks; *Significant differences from the control on 3-week HF medium, $p < 0.01$ (one-way ANOVA and Dunnet test).

plementation of 1 µM 2iP for 1 week (8.1 nodes vs. 7.1), but it was not significantly different from that of the control. Only after 2 or 3 weeks of supplementation at the highest concentration (10 µM), 2iP became significantly inhibiting on node production (3.8 and 3.6 nodes vs. 7.1 on HF medium).

None of the *in vitro*-micropropagated plantlets showed any observable morphological abnormalities.

Ex vitro *transplantation and acclimatization*

Plantlets derived from 2iP treatment which gave the relatively higher node production (8.1), namely 1 µM 2iP for 1 week, as well as plantlets micropropagated on HF medium were addressed to acclimatization. Thirty micropropagated plantlets obtained by culturing three-node explants according to the two mentioned protocols were transplanted. After 3 months from the transplantation, all the 60 transplanted plantlets were successfully acclimatized to *ex vitro* greenhouse conditions and resulted in well-developed plants (Figure 1b). No differences in rooting and acclimatization were observed in the two groups of plants.

RAPD analysis

Four plants for each group ("HF" or "1 µM 2iP for 1 week") of acclimatized plants were analyzed and compared with RAPD profiles of the original donor plant. The 18 selected primers generated a total of 189 bands within 100 and 3250 bp. Each primer produced a number of bands ranging from 3 to 17 (Table 1). The analyzed plants produced 1764 bands and the *in vitro*-micropropagated plants, both from HF and from 1 µM 2iP for 1 week supplemented medium, displayed the same band patterns as the *in vivo* control donor plant. Examples of the obtained RAPD profiles are shown in Figure 3. When the donor plant was compared with three different accessions of *M. quadrifolia*, a certain number of polimorphic bands was instead observed in the RAPD profiles obtained with the primers OPA01,

Table 1. Number and molecular weight range of bands generated as products of RAPD PCR amplification with different primers and DNA from *M. quadrifolia* L., micropropagated by *in vitro* culture.

Primer	Sequence (5′–3′)	No of bands	Size range (bp)
OPA01	CAGGCCCTTC	15	250–2000
OPA03	AGTCAGCCAC	16	270–2100
OPA04	AATCGGGCTG	11	300–2200
OPA05	AGGGGTCTTC	8	570–1720
OPA07	GAAACGGGTG	11	290–2000
OPA09	GGGTAACGCC	14	270–1950
OPA10	GTGATCGCAG	6	320–2360
OPA11	CAATCGCCGT	3	240–2980
OPA12	TCGGCGATAG	10	320–2750
OPA20	GTTGCGATCC	14	440–2080
OPB02	TGATCCCTGG	9	750–2470
OPB08	GTCCACACGG	17	300–2350
OPB10	CTGCTGGGAC	14	260–1900
OPB12	CCTTGACGCA	14	260–2500
OPB16	TTTGCCCGGA	4	460–1800
OPB19	ACCCCCGAAG	3	100–3250
OPB20	GGACCCTTAC	14	380–2070
OPP10	TCCCGCCTAC	6	290–1700
Total		189	100–3250

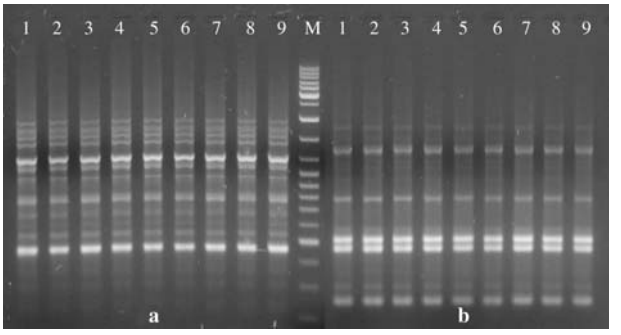

Figure 3. RAPD banding pattern obtained with primers (a) OPA09 and (b) OPB10 in both micropropagated plants and donor mother plant of *M. quadrifolia* L. (M, 100 bp ladder plus; lane 1, mother plant; lanes 2–5, plants micropropagated on medium supplemented with 2iP; lanes 6–9, plants micropropagated on hormone-free medium).

OPA09, OPA10, OPA11, OPB10, and OPP10, showing the existence of an inter-population variability in individuals from different geographic areas (an example is reported in Figure 4).

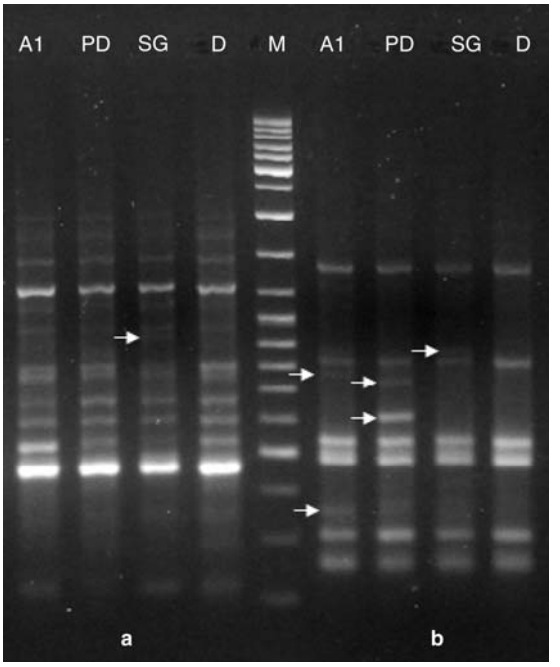

Figure 4. RAPD banding pattern obtained with primers (a) OPA09 and (b) OPB10 in three *M. quadrifolia* samples with different geographic provenience and the donor plant. [M, 100 bp ladder plus; D, donor plant; A1, *M.q.* from S. Nazaro (PR); PD, *M.q.* from Padua Botanical Garden; SG, *M.q.* from S. Giovanni in Persiceto (MO)]. Polymorphic bands are indicated by arrows.

Discussion

Despite its ecological role in maintaining biodiversity and its important role in evolutionary studies, as ferns evolved shortly after emergence of land plants (Qui & Palmer 1999; Kim et al. 2000) and heterospory is a prerequisite for seed evolution, there is a scanty available literature about the *in vitro* culture of *M. quadrifolia* L., maybe due to its scarce economical value. Among the meaningful references, it has been reported that in natural habitats *M. quadrifolia* produces distinct types of leaves referred to as a water form or as a land form and that *in vitro* it is possible to obtain the switch from the water to the land form by the supplementation of abscisic acid to the culture medium or by the application of blue light (Liu 1984; Lin & Yang 1999). More recently, it has been reported that the *in vitro* regenerative response has been enhanced by poor mineral salt medium composition and not by auxin supplementation (Banciu et al. 2009). Thus, in order to enhance the *in vitro* multiplication rate of rhizome explants of *M. quadrifolia*, we investigated the effect of BAP, ZR, or 2iP, structurally different cytokinins. These plant growth regulators, promoting shoot growth from axillary buds even in evolutionary primitive organisms (Mok 1994, and references herein; von Schwartzenberg et al. 2007), have not been assayed yet on this fern, at least as far as we know. Exogenously applied cytokinins are usually extremely effective in improving *in vitro* vegetative micropropagation of a wide variety of plants (George 1993), and they show a different capacity depending on their chemical structure, responsible for the different "strength" of the compound (BAP > ZR > 2iP in order of decreasing activity), and on their concentration (Gaba 2005). It is well known that cytokinins are involved in many developmental processes *in planta*, such as cell division, senescence, and nutrient mobility, and that the endogenous cytokinin homeostasis is regulated by cytokinin oxidases. These enzymes selectively degrade unsaturated N^6-isoprenoid side chain, whereas aromatic cytokinins are resistant to their cleavage, thus causing a different strength in the cytokinin activity (Mok & Mok 2001; Kieber 2002). Cytokinin oxidase activity is highly conserved, as it has been reported either in many higher plant species or in few distantly related lower ones, like the moss *Funaria hygrometrica* Hedw. (Frébort et al. 2011, and references herein). Our experimental data show that the supplementation of cytokinins inhibits the rhizome explants micropropagation of *M. quadrifolia*, the inhibition decreasing with the decreasing strength of cytokinins, without reaching any significant enhancement nevertheless. The severe inhibition observed in the presence of BAP could be an expected result, as it has been already reported that this "strong" cytokinin should be used carefully, adapting the length of the culture period (Fernández et al. 1996; Fernández & Revilla 2003). Moreover, although cytokinins are generally considered as anti-senescence hormones, it has been recently demonstrated that the growth rate of cell suspensions of

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several plant species is reduced while programmed cell death is induced in the presence of high doses of BAP (Carimi et al. 2004, 2005; Zottini et al. 2006). In our experimental conditions, BAP concentrations were quite low, but dealing with ferns we cannot exclude that a similar behavior could be exerted even at low concentrations. Instead, the failure of micropropagation enhancement by ZR and especially by 2iP was unexpected. Thus, we could speculate that the exogenous supplementation of cytokinins induces a supraoptimal concentration of endogenous cytokinins which results in a modification of the fine-tuned homeostasis, extremely important in morphogenetic responses. Perhaps these results should be considered as a typical example of tissue/organ specificity, as rhizome explants do not always behave as stem explants of flowering plants in consequence of exogenous cytokinin supplementation, or it should be due to a *M. quadrifolia* peculiar endogenous cytokinin content in combination with cytokinin oxidase activity. Both should be precisely detected in a further experimental work.

However, as even the exogenous supplementation of auxins is unable to enhance the micropropagation of rhizome explants (Banciu et al. 2009), we could conclude that *M. quadrifolia* is a rather undemanding species, in terms of either mineral nutrition or hormonal supplementation.

Micropropagation from preformed meristems is a common method of propagation for conservation purposes, due to its own low probability of somaclonal variation, but as the *in vitro* culture *per se* and the use of plant growth regulators may generate genetic instability, it is essential to check the genetic fidelity of the micropropagated plants. Thus, we decided to assess the genetic stability of two groups of micropropagated plants: one obtained by HF culture condition and the other obtained by 1 μM 2iP supplementation for 1 week (in which a slight enhancement of mean node number was observed). Among the several DNA markers available, PCR–RAPD analysis summarizes many technical advantages that are particularly significant if a non-economic important genus, as *Marsilea*, is involved. In fact, scanning the whole genome, PCR–RAPD gives the possibility to rapidly evaluate genetic variation in *in vitro*-produced plants. It is remarkable to note that the explants used in the present work originated from donor plantlets subjected to repeated *in vitro* subcultures on HF medium prior to setting up the experimental plan. Albeit the method proved to be sensitive enough to evidence an inter-population variability in *M. quadrifolia* (highlighted by the appearance of polymorphic bands in the RAPD profiles of samples from different geographic origins), no genomic alterations were pointed out in the micropropagated plants. Although lacking of mutation evidence does not necessarily mean that no mutations occurred at all, we can thus conclude that neither the *in vitro* HF multiplication protocol reported here nor the supplementation of cytokinin (2iP) nor the prolonged *in vitro* culture caused genetic instability. Our results are in accordance with those obtained by Banciu et al. (2009), who observed no differences in some isoenzyme activities in *in vitro*-regenerated plants.

This establishes the suitability of the developed protocol for the attainment of true-to-copy micropropagated plants of *M. quadrifolia* that could therefore be reintroduced in its natural environment, as they were also successfully acclimatized to *ex vitro* greenhouse conditions.

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