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In vitro culture of *Pteris vittata*, an arsenic hyperaccumulating fern, for screening and propagating strains useful for phytoremediation

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Abstract — Arsenic contamination of soils and groundwater is at present one of the major emergencies in the world environmental management, and phytoremediation is a promising technology to immobilize or remove contaminants from polluted areas. The first arsenic hyperaccumulating plant, the fern *Pteris vittata*, was discovered only recently. It is very efficient in extracting arsenic from the soil and translocating it into its large fronds, and thus has great potential in arsenic phytoremediation. In this work, by using different hormonal formulations, we developed solid and liquid *in vitro* culture protocols for callus induction and maintenance, as well as for sporophyte regeneration, which are the basic tools for programmes of screening and propagation of selected strains of the fern.

Key words: arsenic, *in vitro* culture, phytoextraction, phytoremediation, *Pteris vittata*.

INTRODUCTION

Arsenic (As), a metalloid belonging to the V group, is known as the poison *par excellence* from ancient times, and is today one of the major public concerns as a universal contaminant of soils and groundwater. Traditionally, remediation of polluted areas relies on *in situ* chemical-physical extraction procedures or on removal of the contaminated soil, both of which are costly and labour intensive (KHAN *et al.* 2000). Recently, some phytoremediation techniques gained attention as an alternative low cost and affordable technology to remove contaminants from soil and water (KHAN *et al.* 2000), among them, phytoextraction makes use of plants to remove contaminants from the soil and concentrate them in the aerial, harvestable biomass (MCGRATH and ZHAO 2003). *Pteris vittata* is a fern that hyperaccumulates As (MA *et al.* 2001); it takes up As very efficiently even from low contaminated soils, can tolerate up to 1,500 mg kg^{-1} As in the soil, and can concentrate the metalloid in the fronds up to 2.3% of the plant dry weight. Moreover, it can produce a large aerial biomass (MA *et al.*, 2001). All of these characteristics make *P. vittata* very suitable for phytoextraction.

The As hyperaccumulating capacity is a constitutive property of *P. vittata* (ZHAO *et al.* 2002) however, variability in this property exists in populations of the fern. Besides, it can be useful to carry out breeding programmes on *P. vittata* by conventional techniques or by genetic engineering (RATHINASABAPATHI *et al.* 2006) in order to obtain strains best suited for phytoextraction. *In vitro* culture can be useful to screen for and propagate clones of the selected strains. *In vitro* culture of ferns is a subject of active research, particularly devoted to the production of ornamental ferns (FERNANDEZ and REVILLA 2003) or to the propagation of endangered species (MANICKAM *et al.* 2003). However, few papers are concerned with the *in vitro* culture of *P. vittata*, and none of them reports well-defined propagation protocols (KATO 1963; 1964; 1965; KWA *et al.* 1991). This paper deals with the preliminary results of a research programme aimed at finding the optimal conditions to initiate and grow callus, as well as to obtain plant regeneration, in *P. vittata*.

MATERIALS AND METHODS

Plants of *P. vittata* were propagated as described in TROTTA *et al.* (2006) from spores col-

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lected in an industrial area of Genova (Italy), and germinated to obtain gametophytes and, subsequently, sporophytes to be used as sources of experimental material. Explants from mature pinnae or croziers of young growing fronds were soaked in distilled water + 1‰ Tween-20 for 1 hour, and then surface-sterilized for 15 min in 1% sodium hypochlorite on a magnetic stirrer. Cultures were made on ½ strength MS medium (½MS) with 0.8% agar in 9-cm Petri dishes, or on the liquid form of the same medium. Liquid cultures were kept on an orbital shaker at 60 rpm. Several hormonal combinations (see Results) were examined to determine the optimal conditions for initiation and indefinite growth of callus and for sporophyte regeneration. About 20 explants were used for all plant materials and hormone combinations. All *in vitro* experiments were carried on in a growth chamber, at 25°C and under continuous light. Some callus and regenerating sporophytes were fixed, embedded in London Resin White, and sectioned according to standard microscopic techniques.

RESULTS

Solid cultures - Mature pinnae strips obtained from young healthy fronds of *P. vittata* did not result in callus production under various combinations of NAA (0.1-5.0 mg l^{-1}) and BA (0.02-1.0 mg l^{-1}), whilst newly formed croziers explanted on medium supplemented with four different concentrations of 2,4-D (0.1; 0.5; 1.0 and 2.0 mg l^{-1}) began to produce callus after two weeks of culture. At the end of 1 month of culture, 1.0 or 2.0 mg l^{-1} 2,4-D yielded 100% of callus initiation and growth, whereas 0.5 mg l^{-1} 2,4-D induced it in about 35% of the explants, and 0.1 mg l^{-1} 2,4-D failed to induce callus proliferation. To explore the effectiveness of 2,4,5-T in callus generation, and to identify the more susceptible parts of croziers to callus induction, we separated the young pinnae from the rachis, and placed them in media containing this compound at concentrations of 2.25; 4.5 or 9.0 µM or 2,4-D 4.5 µM (1.0 mg l^{-1}). After 1 month of culture, callus induction from pinnae was about 65% with 2,4-D or 2,4,5-T 4.5 µM, while it was lower with 2,4,5-T 2.25 µM (about 50%) and 9.0 µM (about 30%). Callus induction from the rachis was 100% with 2,4-D 4.5 µM (Fig. 1A), or 2,4,5-T 2.25 µM and 4.5 µM, while only scarce, senescing callus was induced by 2,4,5-T 9.0 µM. The resulting callus was of the compact type, slow-growing and brownish-green in colour, tending to brown with age.

In order to verify its morphogenetic potential, we transplanted the callus obtained from 1 mg l^{-1} 2,4-D onto media containing 5 µM BA or 5 µM BA + 1 µM NAA. Both hormonal formulations gave rise to vigorous dark green callus, but none of them was able to induce regeneration, nor was effective to this goal the lowering of the hormonal concentration to 1.0 µM BA + 0.1 µM NAA. Successful regeneration was obtained by inverting the hormonal ratio to 0.1 µM BA + 1.0 µM NAA. This formulation generated a fast-growing, friable callus, varying in colour from light to dark green, after 2 weeks (Fig. 1B). This callus, transplanted onto hormone-free medium, regenerated many sporophytic leaves, and, after subdivision of the explants in the same medium, almost 100% of callus pieces rooted after 2-3 weeks. This callus, subcultured monthly on medium containing 0.1 µM BA + 1.0 µM NAA, tended to regenerate even in the presence of this high auxin/cytokinin ratio. To avoid precocious regeneration, we tried a series of four hormonal formulations with a 1:1 auxin/cytokinin ratio, namely BA+NAA 0.1; 0.25; 0.5 and 1.0 µM. The hormonal formulation 0.1 µM BA + 0.1 µM NAA yielded the best results, giving rise to a fast-growing, green callus without signs of organogenesis, which at present is being maintained for further improvement of the culture protocols.

Liquid cultures - Young croziers were explanted on medium with different 2,4-D concentrations (0.1; 0.5; 1.0 and 2.0 mg l^{-1}). After 3-4 weeks, the 0.1 mg l^{-1} 2,4-D treatment produced a callus that, one month later, released cells in suspension. Aliquots of the cell suspension culture were transferred to a hormone-free liquid medium that, after 2 months, gave rise to cell aggregates bearing meristematic centres which began to produce small croziers (Fig. 1 C). Part of the regenerating aggregates were transferred to a hormone-free solid medium or in the presence of 1 µM IAA, NAA or IBA. All treatments yielded 100% rooting in 1-3 weeks, with IBA giving the most precocious results. Some of the rooted sporophytes were transplanted in pots and hardened in 1-2 weeks. The plants developed normally, without any sign of altered morphogenesis.

DISCUSSION

In vitro callus initiation in ferns is generally obtained with 2,4-D (0.1-1.0 mg l^{-1}), using sporo-

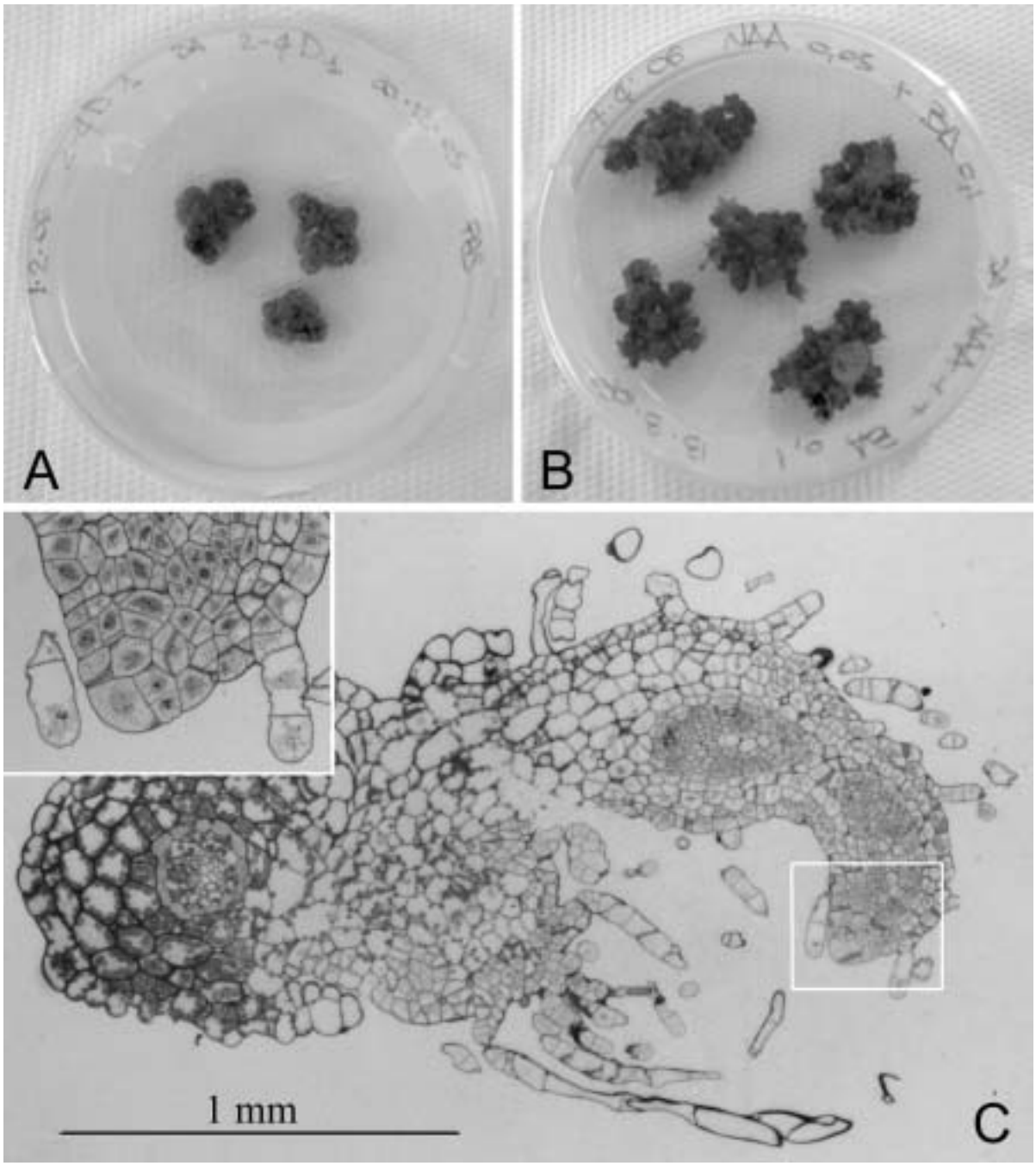

Fig. 1 — A: Callus of *Pteris vittata* obtained after explanting young croziers in ½MS with 1 mg l^{-1} 2,4-D. B: Callus obtained by subculturing in ½MS with 0.1 µM BA + 1.0 µM NAA. C: Histological sections of regenerating cell aggregates grown in hormone-free ½MS; the inset shows the apical meristem.

phytic tissues as explants (Bristow 1962; Kato 1965; Kwa *et al.* 1991; Manickam *et al.* 2003). As a general rule, the younger the explanted material, the more possibilities there are of obtaining a growth response (Kato 1965); therefore, *in vitro*-grown sporophytes are often used as starting material (Bristow 1962; Kato 1965; Kwa *et al.* 1991). However, when the propagation of an adult plant is the goal, it is mandatory to start with older material (Manickam *et al.* 2003). However, our first experiments using mature pinnae failed to produce callus and our best results on callus induction were obtained by treating young croziers with 1 mg l^{-1} 2,4-D (4.5 µM), the same hormonal treatment that was successful in *Pteris cretica* (Bristow 1962). In *P. cretica* as well as in our ex-

periment, 0.1 mg l^{-1} 2,4-D failed to initiate callus, whereas it was successful in the experiment of KATO (1965) on *P. vittata*. Besides this, in our work 2,4-D gave better callus initiation and growth than 2,4,5-T, contrary to the results obtained by MANICKAM *et al.* (2003) on *Diplazium cognatum*, an endangered species of southwestern India. The callus obtained in our experiment was relatively slow growing, of the compact type, brownish-green in colour, tending to brown with age. Maintenance of the callus in 0.1 mg l^{-1} 2,4-D did not change its growth habit; this callus is still in cultivation after about 1 year, and several, approximately bimonthly, subcultures. By transferring to 1 mg l^{-1} 2,4-D young leaves of *in vitro*-grown plants of *P. cretica*, BRISTOW (1962) obtained a very similar callus to that of our experiment, both in appearance and growth potential; it too was subcultured several times in 0.1 mg l^{-1} 2,4-D, and gave rise to sporophyte primordia when transferred to 0.01 mg l^{-1} 2,4-D and sucrose (0.1 or 1.0 g l^{-1}), which developed and rooted in hormone-free medium. On the other hand KATO (1965) explanted young leaves of similarly cultivated plants of *P. vittata* in 0.1 mg l^{-1} 2,4-D, obtaining a callus resembling that of our experiments, which was subcultured in the same medium supplemented with 0.1-1.0 g l^{-1} yeast extract. The subcultured callus gave rise to sporophytic hairs, or rarely to sporophytes, when transferred to hormone-free medium.

To obtain a shoot-type morphogenesis from callus, a high cytokinin/auxin ratio is generally necessary, however in our experiments, we obtained sporophytic morphogenesis with the hormonal formulation 0.1 µM BA + 1.0 µM NAA, which gave rise to a fast-growing, friable callus, varying in colour from light to dark green. The transfer of this callus in hormone-free medium gave rise to numerous sporophytic leaves per callus piece, which rooted after subdivision in the same medium. Moreover, the subculture of this callus tended to give rise to morphogenesis even with a high auxin/cytokinin ratio (1.0 µM NAA + 0.1 µm BA). It is difficult to explain the anomalous behaviour of this plant that produces callus with a high cytokinin/auxin ratio and shoot morphogenesis in the reverse condition. However, as reported by BRISTOW (1962), *P. cretica* regenerated sporophytes even it the presence of 2,4-D alone at low concentrations, and MOREL (1956) obtained sporophytes from a leaf-derived callus of *Adiantum pedatum*, even in the presence of high NAA concentration. Therefore, the hormonal regulation of morphogenesis in *P. vittata* and other fern species needs to be further investigated. A common feature of fern callus is its friable aspect, which we also obtained with 1.0 µM NAA + 0.1 µM BA. A friable callus of *P. vittata* was also obtained by KATO (1965), who then put the callus in liquid medium to establish a suspension culture of cells and callus fragments. Likewise, we obtained fragmentation and growth of the callus when transferred in liquid medium (unpublished results). However, we obtained a true cell suspension by explanting croziers directly in a liquid medium added with 0.1 mg l^{-1} 2,4-D. The cell suspension gave rise, when transferred to hormone-free medium, to cell aggregates that originated meristematic centres and then sporophytes, with a modality very similar to that reported by BRISTOW (1962) in *P. cretica*.

In conclusion, in this preliminary work on *P. vittata,* we laid the foundations for the initiation and indefinite growth of the callus, as well as for the regeneration and rooting of sporophytes, which are the basic tools for initiating a programme of *in vitro* screening for and propagation of selected strains of the fern.

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