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BRIEF COMMUNICATION

Microwave treatment induced mutations and altered gene expression in *Vigna aconitifolia*

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Abstract

Primary leaf explants of aseptically grown seedlings of moth bean [*Vigna aconitifolia* (Jacq.) Marechal] immersed in water or not were treated in microwave oven (2450 MHz, 800 W cm^{-2}) for 1, 3, 5 and 7 s before culturing. Callusing and shoot emergence from these explants were enhanced up to microwave exposure lasted 5 s while longer treatment of water-immersed explants delayed callusing. One polypeptide (26.6 kD) was up regulated in the callus derived from microwave treatment in water-immersed explants. RAPD analysis detected alteration in DNA sequences due to microwave treatment in water-immersed explants for 7 s. The frequency of mutation was 1.6 % (4 bands out of 248) over all the cultures analyzed and the same was 13 % (4 bands out of 31), if amplicons generated at 7 s treatment alone were considered.

Additional key words: *in vitro* culture, moth bean, RAPD.

Microwaves have various effects on biological systems at whole organism, tissue, cell and molecular level (Roux *et al.* 2006, Hamada 2007). Most of these studies were focused on very weak (> 0.5 mW cm^{-2}) and low frequency magnetic fields with a view to find out their toxic or side effects. Nevertheless, microwave enhanced germination, plant height and fresh mass was observed (Aladjadjiyan 2002, Belyavskaya 2004, Racuciu *et al.* 2006). In a microwave oven, microwaves generate rotation in dielectric molecules like water under the influence of electromagnetic field resulting in heating of the system. It is envisaged that this rotation may destabilize bio-molecules including DNA. Keeping this in view, the present investigation was carried out to determine the effect of strong microwaves in relation to surrounding water, on regeneration potential, gene expression and genetic stability in moth bean (*Vigna aconitifolia),* a drought tolerant pulse crop amenable to various tissue culture techniques.

Seeds of moth bean [*Vigna aconitifolia* (Jacq.) Marechal] cv. RMO-40 were surface sterilized by 0.1 % $HgCl_2$ and grown in test tubes on filter paper bridges. Seven to eight days old *in vitro* seedlings were divided into three sets. In the first set, the entire seedlings were immersed in water while the seedlings of the second set were kept in air. Microwave treatment (2450 MHz, 800 W cm^{-2}) was given to the seedlings of both sets 1, 3, 5, and 7 and 9 s using microwave oven (model *GMS 22A*, *Godrej*, India). The third set of seedlings (without microwave treatment) was used as control.

Primary leaves from treated as well as control seedlings were cut and inoculated on MS medium supplemented with 3 mg dm^{-3} benzylaminopurine (BAP) and 1 mg dm^{-3} indoleacetic acid (IAA). Proximal and distal ends of leaves were cut to avoid preexisting meristem. The cultures were incubated at temperature of 27 ± 0.5 °C and 14-h photoperiod (fluorescent tubes, the average irradiance of 68 μmol m^{-2} s^{-1} at bench level). Survival percentage of explants, number of days to callus induction, shoot emergence and number of shoots produced per explant were recorded periodically.

Total proteins were extracted from leaves of seedlings 30 min after treatment, after 48 h of incubation and after 45 d at callus induction. 500 mg of tissue was ground in 0.8 cm^3 of buffer [50 mM Tris-HCl, urea (8 %), sodiumdodecyl sulfate (SDS; 2.0 %), glycerol (10 %),

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Abbreviations: BAP - benzylaminopurine, IAA - indole acetic acid; PAGE - polyacrylamide gel electrophoresis; RAPD - random amplified polymorphic DNA; SDS - sodiumdodecyl sulfate.
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β-mercaptoethanol (5 %)] using chilled mortar and pestle. The ground material was collected in Eppendorf tube and was centrifuged at 25 000 *g* for 10 min. Samples denatured by boiling for 5 min were transferred on 12 % polyacrylamide gel. The gels were stained by Coomassie brilliant blue R250 and silver (Sambrook *et al.* 1989).

Random amplification of polymorphic DNA (RAPD) analysis was conducted using purified DNA from regenerated plants. Plant material was homogenized in liquid nitrogen and DNA was extracted by the method of Doyle and Doyle (1990). The quantified DNA was diluted to final concentration of 25 μg cm^{-3} and was stored in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at -20 °C. RAPD analysis was done by using 10 primers of OPG series obtained from *Operon Technologies* (Alameda, CA, USA). PCR reactions were performed in final volume of 25 mm^3 containing 10× assay buffer, 0.5 units of Taq DNA polymerase, 200 μM each of dNTPs (*Bangalore Genei*, Bangalore, India), 10 pmol per reaction of random primers and 50 ng of template DNA. The PCR was performed in *Biometra* (Germany) thermocycler with cycling parameters; denaturation at 94 °C for 5 min, primer annealing at 37 °C for 1 min, primer extension at 72 °C for 2 min; then 2 - 43 cycles at 94 °C for 1 min followed by 7 min extension at 72 °C. Following amplification, the PCR products were loaded along with a 200 bp ladder on 1.2 % agarose gel (*Merck,* Germany) prepared in 0.5× TBE buffer (45 μM Tris, 45 μM borate and 1 μM EDTA) containing 0.5 μg cm^{-3} of ethidium bromide. The amplified products were electrophoresed for 5 h at 50 V. After separation, the gel was viewed under UV radiation, photographed and the appearance of novel bands was recorded.

Both the types of treatments, water immersed and non-water immersed, were injurious to explants and mortality increased with increasing treatment duration with complete mortality at 9 s. Partial or whole tissue death was observed for all the treatments except for 1 s, with a reduction in survival to 20 % in water-immersed and to 25 % in non-water immersed treatments for 7 s (Table 1). However, the tissue response in the form of swelling, observed after 24 h of incubation, was better in the treated explants than in controls, especially when explants from water-immersed seedlings were used. The callusing was reported earlier in all the treated plants than in controls with exception of water-immersed seedlings treated for 5 and 7 s, though these initially showed better tissue swelling. Non-water immersed explants showed earlier callusing than their water-immersed counterparts for all treatment periods. Similarly, treated explants developed shoots earlier than control, however, the numbers of shoots were negatively affected by microwave treatment (Table 1).

The dose dependent effect of microwaves observed in the present study is in conformity with other studies (Alexander and Doijode 1995, Carbonell *et al*. 2000, Celestino *et al*. 2000). Likewise, heat treatment has been shown to affect regeneration and further growth in both ways, *i.e.* negatively (Burbulis *et. al.* 2004) and positively (Morini *et al.* 2004). In the present study, rise in temperature of water-immersed explants from 27 (control) to 29 and 30 ºC for 1 and 3 s showed negligible influence on regeneration. However, 5 and 7 s treatment where the rise in temperature was considerable (to 38 and 48 ºC) suggests the influence of microwaves *per se*. Elevated temperature seems to be associated with tissue death that increased abruptly at 5 and 7 s treatments (Table 1).

Table 1. Effect of microwave treatment on regeneration of moth bean. Means ± SD, $n = 20$.

Treatments	Time [s]	Explants survived [%]	Time to callus induction [d]	Time to shoot emergence [d]	Number of shoots [explant^{-1}]
Control	0	100	14.80±1.03	59.10±4.65	3.05±0.94
Water immersed	1	90	14.56±1.13	54.33±4.64	1.94±0.80
	3	85	14.63±1.92	52.63±4.96	1.63±0.74
	5	50	16.43±1.62	49.57±2.94	1.20±0.45
	7	20	16.50±1.29	52.67±6.43	1.25±0.50
Non-water immersed	1	100	13.70±1.16	58.90±4.20	2.25±0.55
	3	90	13.11±1.27	48.22±4.12	2.00±0.71
	5	40	12.60±0.55	52.25±4.19	1.20±0.45
	7	25	13.75±0.96	53.50±6.19	1.25±0.50

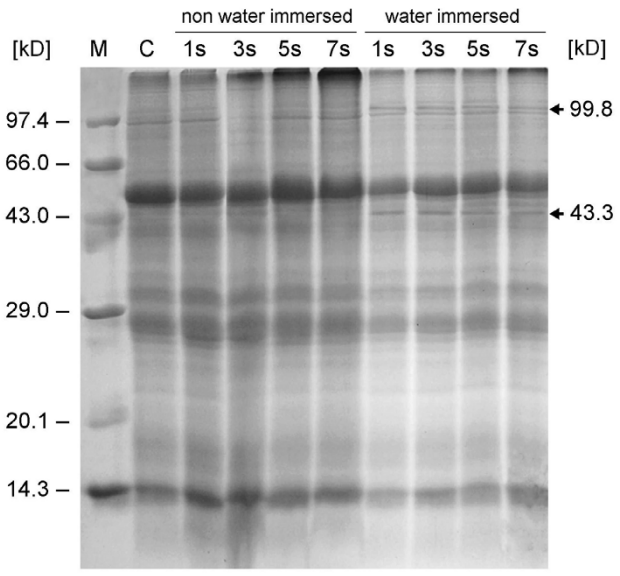

Fig. 1. Leaf protein pattern immediately after microwave treatment to non-water immersed and water immersed moth bean seedlings and lasted 1, 3, 5 and 7 s. M - protein mass marker, C - control.

The gene expression after 30 min of treatment was affected differentially in water immersed and non-water immersed explants (Fig. 1). Two polypeptides (99.8 and

44.3 kD) were found up regulated only in water immersed explants and the 44.3 kD polypeptide was up regulated only with shorter treatments (1 and 3 s). Though, not well resolved, the protein bands of high molecular mass appearing just below the wells were found to increase with increasing dose in both the types of treatments. While earlier pair of polypeptides seems to be influenced by microwave *per se,* later high molecular mass proteins prominent at 5 and 7 s treatments with considerable rise in temperature could belong to HSP family that includes heat or dehydration inducible proteins (Vishwanathan and Chopra 1996). As observed for regeneration parameters, gene expression was also differentially affected in water immersed and non-immersed treatments. The damaging effect on cell membrane and associated change in permeability leading to variation in solute concentration could be the reason for differential effect of surrounding water influencing the gene expression and regeneration after microwave treatment.

Marked effect of microwaves on gene expression was reported after 48 h of incubation especially in explants from water-immersed seedlings (Fig. 2). While expression of only two proteins (122.2 and 58.3 kD) was found up regulated in non-water immersed explants, expression of four proteins (66, 37.9, 35.3 and 33.8 kD)

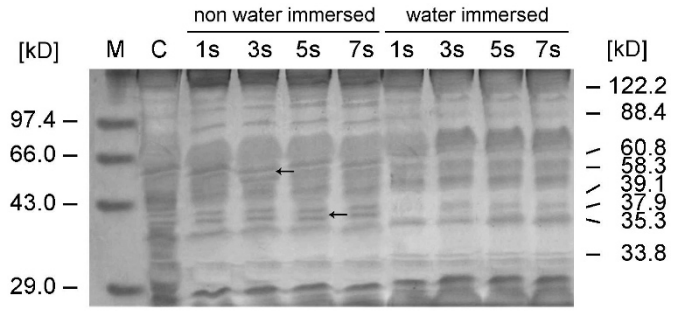

Fig. 2. Leaf protein pattern 48 h after microwave treatment to non-water immersed and water immersed seedlings and lasted 1, 3, 5 and 7 s. M - protein mass marker, C - control.

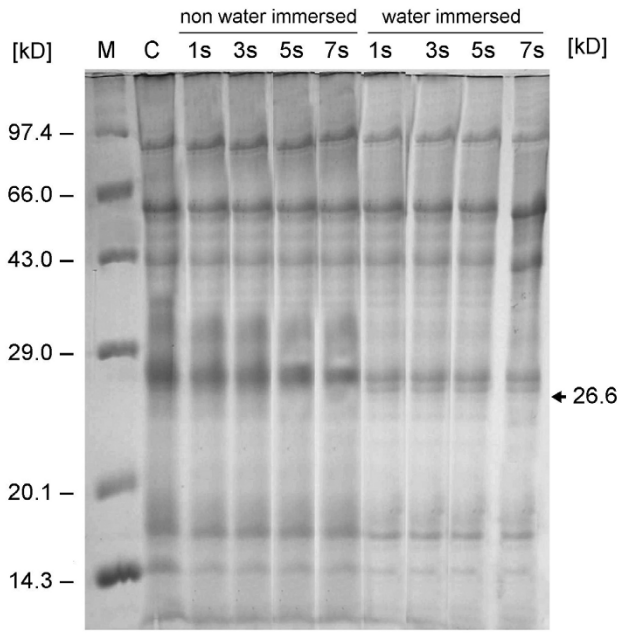

Fig. 3. Protein pattern in callus regenerated (45 d) after microwave treatment to non-water immersed and water immersed seedlings and lasted 1, 3, 5 and 7 s. M - protein mass marker, C - control.

was up-regulated in water-immersed explants along with down-regulation of three proteins (88.4, 60.8 and 39.1 kd). None of them was common in water immersed and non-water immersed seedlings. Moreover, one

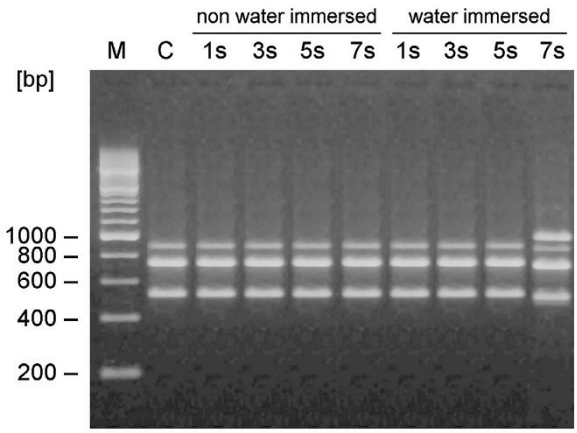

Fig. 4. RAPD profile (primer OPG-3) showing novel bands in regenerating plantlets after microwave treatment to non-water immersed and water immersed seedlings and lasted 1, 3, 5 and 7 s. M - ladder, C - control.

polypeptide (26.6 kD) was differentially up-regulated in callus developed from water-immersed explant after 45 d of incubation (Fig. 3). Considering the consistency in banding pattern for all the treatment durations for a particular type of treatment, it could be suggested that most effects are due to microwave *per se* and not due to a thermal effect as was suggested previously (D'Andrea *et al.* 2003). This study indicated the long lasting direct effect of microwaves on gene expression with temporary (protein profile for 30 min after treatment) effect of

Table 2. Mutagenic effect of microwaves demonstrated by appearance of novel bands in regenerated plantlets from treated explants in month bean.

Primer	Sequence (5'→ 3')	Total number of bands	Presence of novel band
OPG-2	GGCACTGAGG	3	0
OPG-3	GAGCCCTCCA	4	1
OPG-4	AGCGTGTCTG	7	2
OPG-6	GTGCCTAACC	2	0
OPG-7	GAACCTGCGC	4	0
OPG-11	TGCCCGTCGT	2	0
OPG-13	CTCTCCGCCA	4	0
OPG-17	ACGACCGACA	5	1

associated increase in temperature.

Another interesting feature was the polymerase chain reaction (PCR) amplification of novel bands in the plantlets developed from treated explants (Fig. 4). All these were observed at treatment lasted 7 s and in water immersed explants. This makes a very high frequency of mutagenesis, 13 % (4 out of 31 bands). This mutagenic frequency would be only 1.6 % (4 bands out of 248) if all the plantlets developed from treated explants were considered. Such a high mutation rate especially with more than one novel band for a single primer could be expected with gross chromosomal aberrations that have been indicated to be produced more frequently after microwave treatments (Pavel *et al*. 1998).

The present investigation proposes the use of microwave ovens for *in vitro* culture manipulation and mutagenesis as an alternative to chemical mutagens and γ-chambers. The clear indication provided in the present study in favour of microwaves *per se* may help to develop a system to resolve the effect of microwaves on biomolecules, tissues, organisms and gene expression.

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