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Microwave seed treatment reduces hardseededness in *Stylosanthes seabrana* and promotes redistribution of cellular water as studied by NMR relaxation measurements

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Summary

Seeds of *Stylosanthes seabrana* possessing seed coat and physiological dormancy were exposed to microwave fields with a dosage between 140 and 1400 W g^{-1} FW. The treatment improved germination percentage from 7% in the untreated control to 45% when seeds were treated with a microwave energy between 840 and 1260 W g^{-1} FW. Seed leachate conductivity also increased by 28% in treated seeds compared to the control, indicating the loosening of the seed coat membrane. However, higher dose of energy (1400 W g^{-1} FW) reduced germination by increasing the number of abnormal seedlings. The imbibition rates were significantly higher in treated seeds than in untreated seeds. Microscopic scanning of the seed coat using a Deltapix (Infinity X) camera system showed disintegration of seed coat in treated seeds that would have helped water uptake. The transverse relaxation time (T_2) of seed water and its components was measured *in vivo* using nuclear magnetic resonance spectroscopy to explain the changes in seed water status during germination in microwave treated and untreated seeds. The results showed that in microwave treated seeds the fraction of least mobile water (bound water) which hydrates the macromolecules appeared much earlier than in the untreated seeds. This enhanced hydration would have initiated various metabolic activities related to germination process. In conclusion, microwave treatments can soften the seed coat, enhance imbibition rate and reduce hardseededness in seeds of *Stylosanthes*.

Introduction

Stylosanthes is regarded as an important range legume for the humid to semi-arid tropics. It has a major role in waste land development as it can restore soil fertility and improve soil physical properties. Therefore, in India, *Stylosanthes* has been identified as an important species in programs supported by the National Wasteland Development Board, the National Afforestation and Eco-Development Board, State Departments of Agriculture, Animal Husbandry and Forestry and the Grassland and Fodder Research Institute of Indian Council of Agricultural Research, India. Recently, an evaluation of a new species of *Stylosanthes*, namely *S. seabrana* B. L. Maass & 't Mannetje, at different Indian locations has indicated the potential of this species for wider application in the country (Chandra *et al.*, 2006). The major problem with this fodder legume is the establishment of good field stand owing to its low germination as the seeds show a seed coat dormancy that restricts the moisture availability to the embryo.

This can be overcome by scarification, acid or heat treatment. The presence of naturally occurring germination inhibitors also results in delayed germination. These inhibitors can be degraded in most cases by any stressing factor such as high temperature (Holm, 1972) or by soaking the seeds in thiourea (Delatorre and Barros, 1996) which can induce ethylene production by the seeds to counteract the inhibitors.

Microwave and radio-frequency (RF) dielectric heating have been used for controlling stored-grain insects (Nelson, 1996; Wang and Tang, 2001). In wheat, no detrimental effect on germination was observed due to short exposures to RF or microwave field (Nelson and Walker, 1961). Therefore, experiments were conducted using different microwave energies to determine if this treatment could overcome the problem of restricted imbibition in *S. seabrana*. Nuclear magnetic resonance (NMR) spectroscopy offers a non-destructive and non-invasive method for characterization of water status in many biological tissues including seeds (Brosio *et al.*, 1992; Fukoka, *et al.*, 1994; Krishnan *et al.*, 2003). Longitudinal and transverse relaxation behaviour of water protons can be investigated to describe the compartmentation and transport of water in tissues. This technique was used to detect the enhanced uptake of water and its compartmentation in microwave treated seeds of *S. seabrana*.

Materials and methods

Seeds of *Stylosanthes seabrana* were obtained from Crop Improvement Division of Indian Grassland Research Institute, Jhansi and cleaned thoroughly. They were equilibrated over $CaCl_2$ in a desiccator and conditioned to a moisture content of 6% on DW basis. Seed samples of 0.5 g in mini Petri dishes (5 cm diameter) were placed for 1 minute in a domestic oven (Padmini make, India) with maximum microwave output of 700 W at 2.45 GHz frequency. Doses of microwave treatment of 70 to 700 W in steps of 70 W were given regulated by an analog control system. There were three replications for each treatment and the treatment energy was expressed in watt per gram of seed fresh weight (W g^{-1} FW). The germination percentage was determined by placing three replicates of 50 seeds each between moist germination papers in an incubator at 25°C and after 14 days the normal and abnormal seedling were counted.

To determine the seed leachate conductivity, 0.2 g seeds were placed in a vial and 20 mL distilled water was added. The vials were kept in an incubator at 25°C for 24 h. The conductivity of leachates was measured with a digital conductivity meter (Systronics Model 304, India) by subtracting the electrical conductivity of distilled water from that of seed leachate and expressed as m S cm^{-1} g^{-1} FW.

Based on the germination test results, two microwave treatments (980 and 1260 W g^{-1} FW) giving highest germination and the control were selected for imbibition studies and microscopy. For imbibition studies, seeds were hydrated at 25°C by placing them on two layers of Whatman filter papers on large covered Petri dishes (16 cm diameter). After different imbibition periods, seeds were taken and the excess of surface moisture was removed between layers of tissue paper. The seed moisture content was determined by oven drying at 95°C to constant weight (Walters, 1998). Moisture content was calculated as the mean of three replications on a dry seed basis.

For NMR measurement, seeds were filled to about 1.5 cm height in the NMR tube (10 mm diameter) which was then weighed accurately and sealed immediately and placed in the probe of NMR Spectrometer (Bruker NMS 120, Germany). Spin-spin relaxation time T_2 was measured by the Carr-Purcell-Meiboom-Gill method (Snarr and Van As, 1992) at 20 MHz. Each measurement had the following settings: data points 150, pulse separation 0.5 ms, dummy echo 3 and scans 15. Gain was adjusted to maximize the signal to noise ratio. The data points were fitted using the built-in Expspel program with the single exponential decay observed in the CPMG sequence. Two replicate samples were measured.

In biological systems, including seeds, multi-exponential relaxation decay curves are generally observed, clearly indicating the presence of three components with different relaxation times. According to Ratkovic (1987), three water components of the seed system are identified by the spin-spin relaxation times T_{2a}, T_{2b}, T_{2c}. The component T_{2c} accounts for the hydration water of macromolecules and is tightly bound, T_{2b} for the cytoplasmic bulk water with lower mobility and T_{2a} for the extra-cellular free water. The three components of spin-spin relaxation times are given by the equation

$$M_t = C_a \{\exp(-t/T_{2a})\} + C_b \{\exp(-t/T_{2b})\} + C_c \{\exp(-t/T_{2c})\},$$

where C_a, C_b, and C_c are related to the relative population of three components (Di Nola *et al.*, 1991; Brosio *et al.*, 1992). The components of spin-spin relaxation were analysed by using least square fit analysis in the region of limits specified, based on t values (x-axis) until the plotted curve showed a visible curvature change, using a self written C++ program. The general procedure of exponential peeling of curve decomposition was followed by locating the slowest relaxing fraction from the curve and subtracting this fraction from the observed data (figure 1) (Snarr and Van As, 1992; Nagarajan *et al.*, 2005).

High resolution (>150 pixel mm^{-1}) macro-photography images of the seed coat surface were captured with a Deltapix (Infinity X) digital camera (Deltapix, Denmark). The camera was fitted on a stand with a light source and lens with magnification of 60X. The image was stored with the help of the software package DPX view LE (Deltapix, Denmark).

Results

Normal seedling percentage improved from 7% in the untreated control to 45% when seeds were treated with microwave energies between 840 and 1260 W g^{-1} FW (figure 2). Below this range of energy, the germination, though higher than the control, was only 21-24%. Above this range, the percentage of normal seedlings decreased with a corresponding increase in abnormal seedlings. Seed leachate conductivity also increased significantly in treated seeds compared to the control, and was highest in seeds treated with 1400 W g^{-1} FW (figure 2). Imbibition of treated and control seeds showed the typical three phase curve of water absorption (figure 3). The first phase of rapid absorption (until 16 h) and the second phase of very slow absorption (until 54 h) were similar in treated and control seeds. But in the third phase, a more rapid water uptake was observed only in treated seeds. In the control seeds, the water content increased from the initial level of 0.06 to 0.92 g g^{-1} DW during phase I and from 1.25 to 1.64 g g^{-1}DW during phase III, respectively. In microwave treated seeds, the water increase rose from 0.06 to 1.25 g g^{-1}DW during phase I and from 1.56 to 2.70 g g^{-1} DW during phase III, respectively.

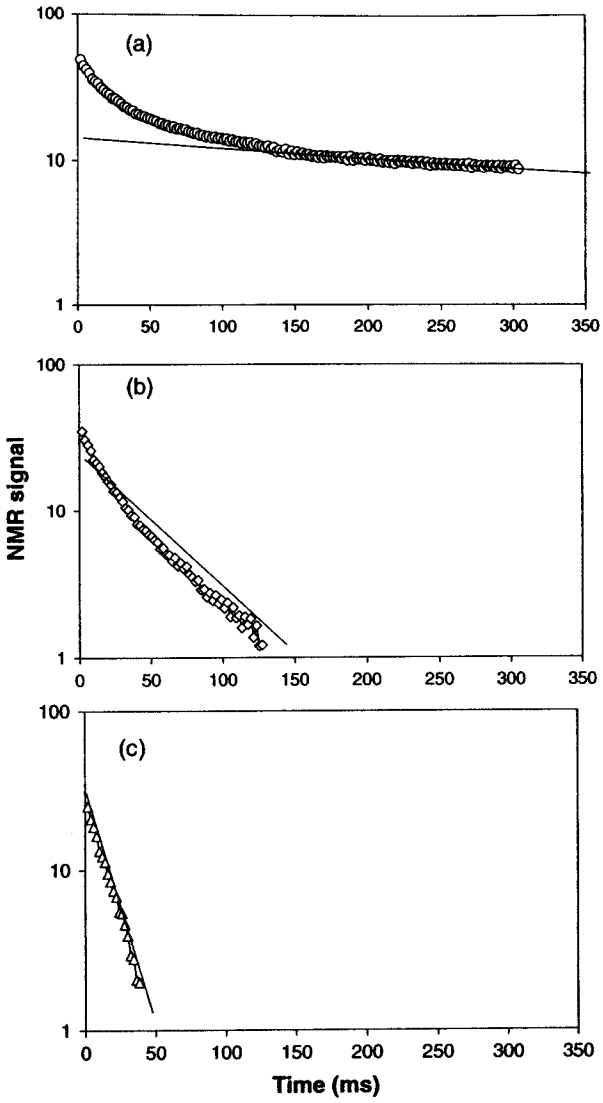

Figure 1. Decomposition of the spin-spin relaxation time T_2: a, Semi-logarithmic plot of the observed transverse magnetization decay curve. Also shown is the straight extrapolated line of the slow–decaying component T_{2a}; b, Semi-logarithmic plot of the transverse magnetization decay curve of the sum of fractions T_{2b} and T_{2c}, which is the vertical difference between the observed curve and the straight T_{2a} line reported in a). Also shown is the straight extrapolated line of the component T_{2b}; c, Semi-logarithmic plot of the transverse magnetization decay curve of fraction T_{2c} which is the vertical difference between the T_{2b} + T_{2c} curve and the straight T_{2c} line reported in b).

The component analysis of the seed water showed interesting results. In dry seeds it was not possible to resolve through this analysis into the three components with confidence, due to a low proton signal. Brief hydration of seeds led to better resolution so that relaxation times and the fractions of protons in different magnetic environments could be computed. The two components of relaxation times T_{2a} and T_{2b} increased initially with imbibition time albeit at different rates in treated and control seeds, stabilized during phase II of imbibition and then again increased after germination, the increase being highest only in treated seeds

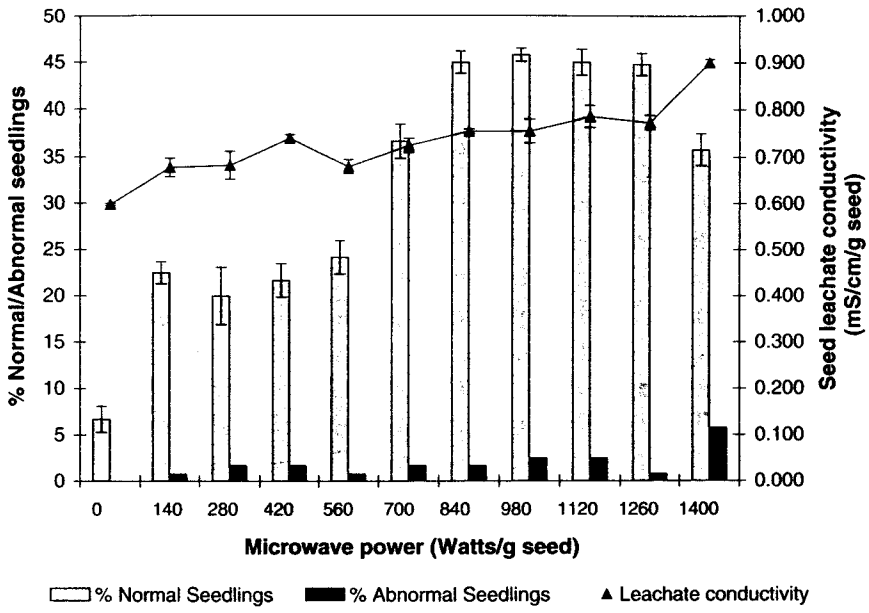

Figure 2. Effect of microwave radiation on the percentages of normal and abnormal seedlings and seed leachate conductivity in *S. seabrana*. LSD (P=0.05) for arcsine transformed percent-values of normal and abnormal seedlings are 7.6 and 0.41, respectively and 0.091 for leachate conductivity.

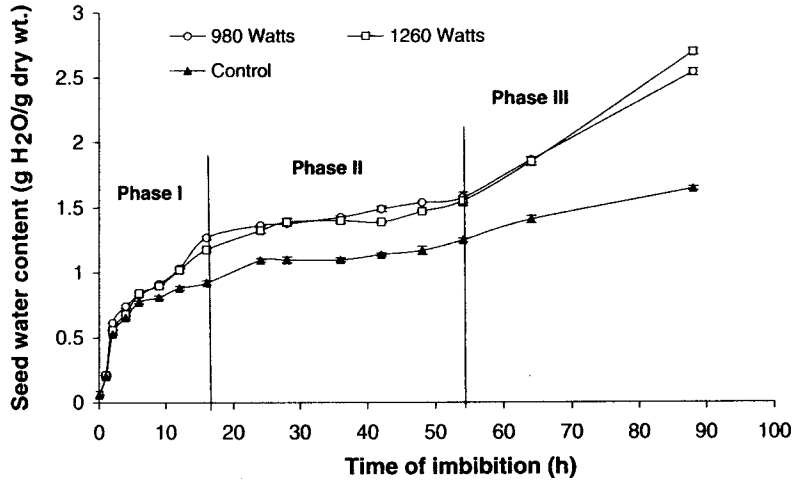

Figure 3. Time-course of water content increase during imbibition at 25°C of microwave treated (980 and 1260 W g^{-1} FW) and untreated *S. seabrana* seeds.

(figure 4 a-b). During the lag phase in control seeds the relaxation time T_{2a} of the slow relaxing component was greater (figure 4 a), while T_{2b} was lower than in treated seeds (figure 4 b). The fast relaxing component associated with least mobile water fraction appeared after 22 h of imbibition in treated seeds whereas it appeared only after 60 h of imbibition in control seeds. The relaxation time (T_{2c}) of this fraction of water in control was lower than that of treated seeds. Compared to control in treated seeds, the fraction of water in bound and less mobile (cytoplasmic) form together, was greater than the fraction of water in more

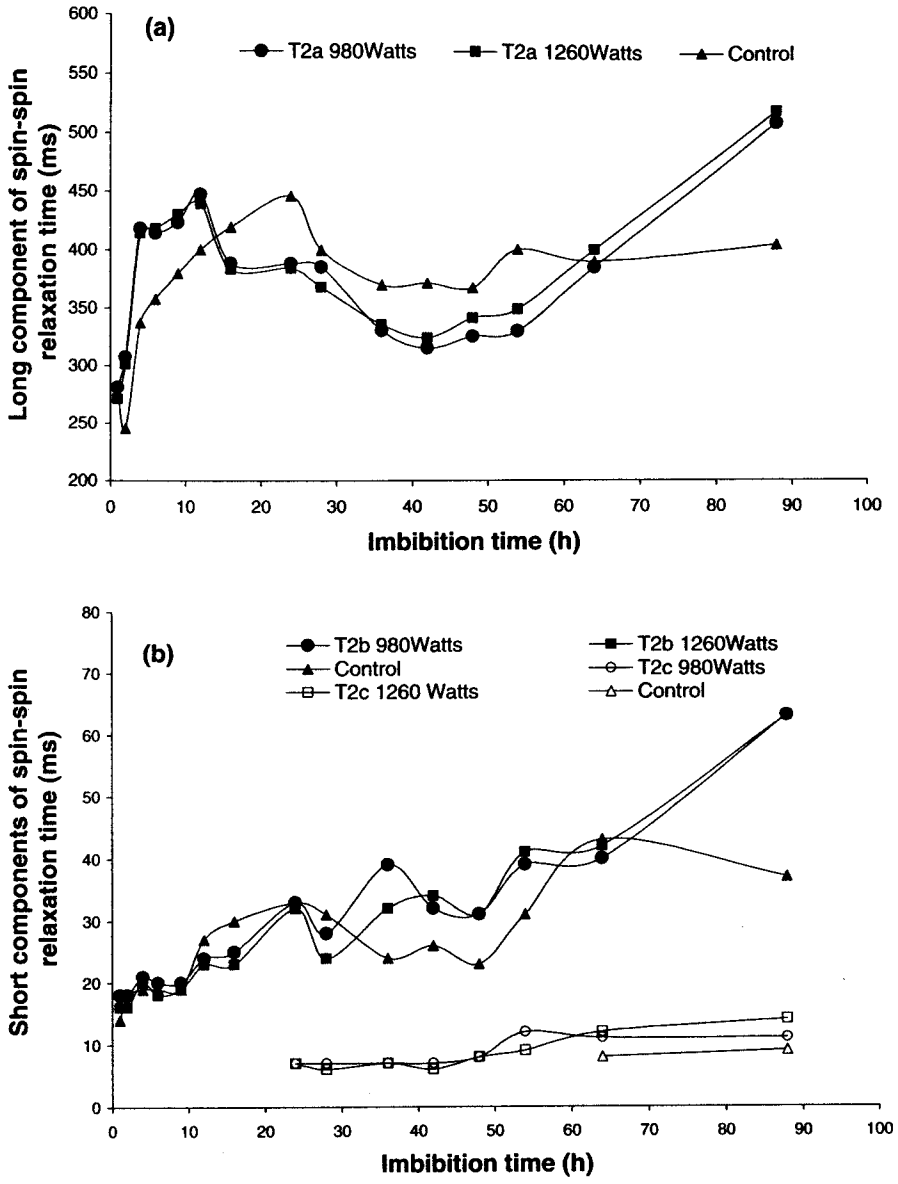

Figure 4. Changes in the long (T_{2a}; a) and short (T_{2b} and T_{2c}; b) components of spin-spin relaxation time in microwave treated (980 and 1260 W g^{-1} FW) and untreated *S. seabrana* seeds during germination.

mobile (extracellular) form. This could be observed after 16 h after imbibition (figure 5 a-c). After 88 h of imbibition, there were only two components, the cytoplasmic and the bound species, of seed water in treated seeds following the onset of the germination process. High resolution, magnified photographs of the seed coat morphology are shown in figure 6 a-c. In microwave treated seeds it was noted a rupturing of the seed coat and the subsequent radicle emergence following imbibition.

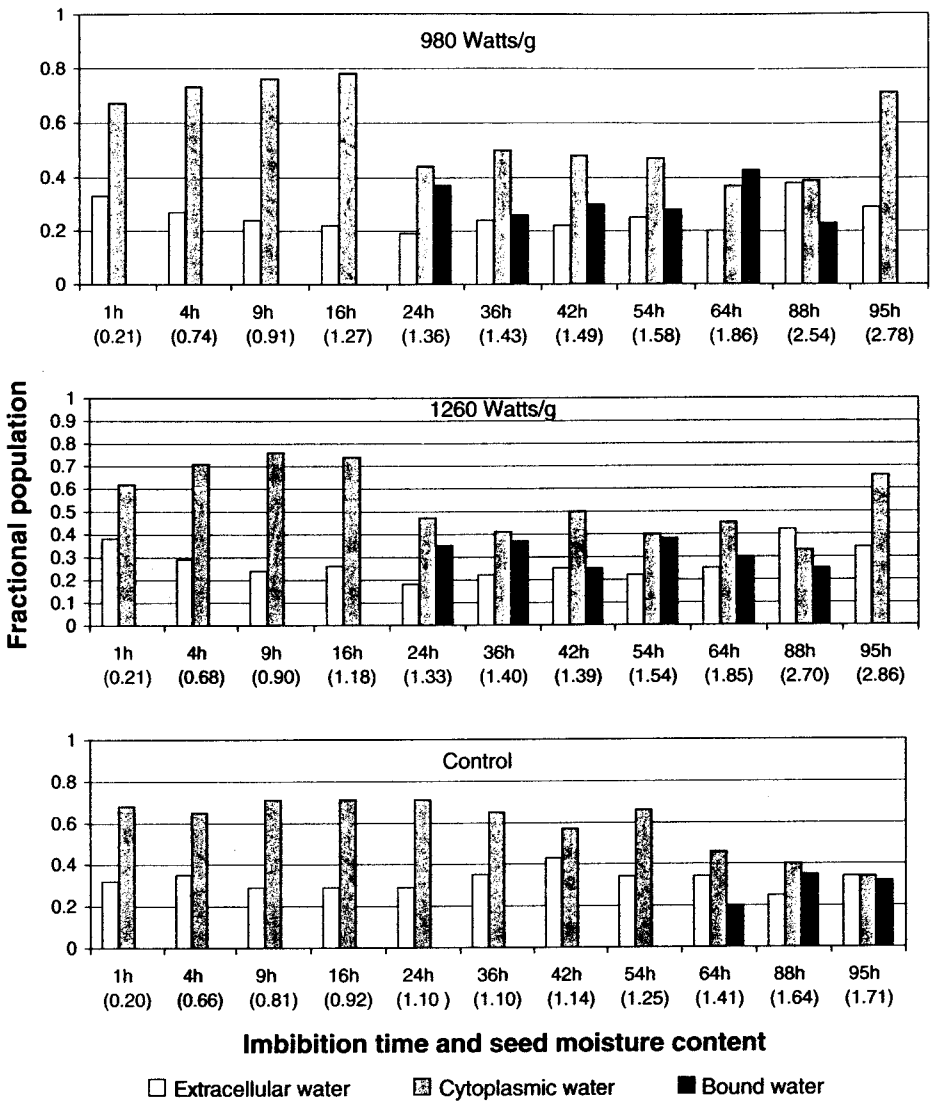

Figure 5. Fractional population of water protons of varying mobilities during germination of microwave treated at 980 W g_{-1} FW (a), at 1260 W g-1 FW (b), and untreated (c) *S. seabrana* seeds. The plain, grey and black coloured bars denote protons associated with more (extracellular water), less (cytoplasmic water) and least (bound water) mobilities, respectively. The seed moisture content at different times of imbibition are shown between brackets under X axis.

Discussion

Germination of *Stylosanthes seabrana* seeds is affected by hardseededness and physiological dormancy. Methods like scarification with sand paper, acid and hot water treatments remove hardseededness by cracking the seed coat, allowing imbibition. Microwave radiation appears to allow breaking up of the seed coat as shown by the magnified photographs. The increased conductivity of seed leachates of treated seeds over control indicated that the seed coat

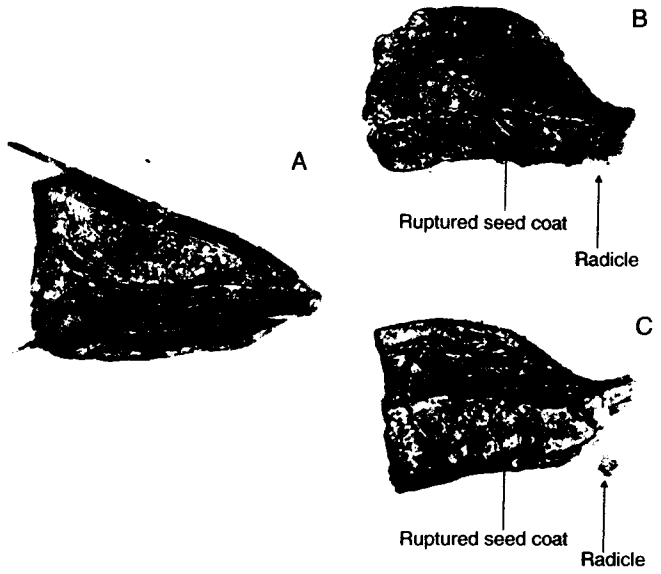

Figure 6. Magnified photographic images of *S. seabrana* seeds: a, untreated seeds; b and c, seeds treated with 980 or 1260 W g^{-1} FW, respectively, microwave energy showing rupturing lines on the seed coat and emerging radicle.

membrane had become permeable to solutes such as sugars, organic acids, ions, and amino acids that are released once seeds start to imbibe water (Smith and Berjak, 1995). Low leakage from control seeds shows that the uptake of water is restricted by the intact seed coat which acts as a barrier to the efflux of solutes. Apart from causing physical aberration to the seed coat, microwave radiation also produces dielectric heating of the seed. This may also partially remove physiological dormancy. A 1400 W g^{-1} FW radiation dose, however, decreased the percentage of normal seedlings with increasing abnormal seedlings and seed leachate conductivity. In germinating seeds, the leakage of electrolytes is immediately minimized, due to re-establishment of membrane integrity to prevent further leakage. Relatively highest leachate conductivity and percentage of abnormal seedlings in seeds exposed to 1400 W g^{-1} FW imply that the re-establishment of membrane integrity is hampered resulting in damage to inner cell membranes. There is a threshold value for the microwave radiation dose, beyond which it may be non effective.

As described by Bewley and Black (1985), the kinetics of seed water uptake shows clearly three distinct phases of water absorption, namely, rapid hydration (phase I), a lag phase (phase II) and a fast hydration resumption due to germination and radicle protrusion (phase III). Phase I imbibition, which is largely a consequence of matric forces, and phase II imbibition, due to hydration of food reserves, take place rapidly in treated seeds and very slowly in those untreated because of slow permeability to water. In control seeds, the third phase was not discernable due to germination inhibition.

The components of NMR relaxation time T_2 of seed water and its distribution within the seed explain the possible mechanism by which the germination of microwave treated seeds improved. The data indicate the presence of three different populations of protons, each with different relaxation times and mobility. Extracellular water is characterized by

high relaxation times, intracellular bulk water by low relaxation times and bound-structural water by very low relaxation times (Di Nola *et al.*, 1988; 1991; Brosio *et al.*, 1993; Foucat *et al.*, 1993). The transverse relaxation time of seed water protons is related to translational and rotational motion of water molecules in homogeneous systems (Samuilov *et al.*, 1979). Higher relaxation time implies greater randomness of water molecules and better availability for involvement in metabolic activities (Lewin, 1974). However, in heterogeneous systems such as seed tissues, the mobility of water molecules is modified by the effects of diffusion and morphology (Belton and Colquhoun, 1989). The higher values of T_{2a} in phase III and T_{2b} in phase II and III of imbibition in treated seeds may be indicative of higher molecular mobility of that fraction of water and hence better availability for metabolic activities related to the germination process.

The fractional population of different components of seed water indicated that in both control and treated seeds imbibed water was initially distributed between extracellular water and cytoplasmic bulk water. But as the macromolecules of the embryo and endosperm tissue became hydrated in treated seeds, a third component of hydration water with less mobility appeared at 24 h imbibition. The synthesis and mobilization of proteins with seed hydration may be responsible for this water fraction. The proportions of this macromolecular hydration water and that of cytoplasmic bulk water together account for 80% of seed water and seem to play an important role in the germination process. Krishnan *et al.* (2004) reported complete disappearance of the bulk water fraction in non-viable soybean seeds during imbibition. Bacic *et al.* (1992) have shown that the embryo water fraction had the shortest T_2 values, essential for maintaining seed viability. The appearance of only two components of water after 88 h indicates the exchange of water in different compartments by rearrangement of membrane permeability during germination and the formation of vacuoles in association with embryo growth. This trend was not observed in control seeds at corresponding time together with the detection of the third component of water with least mobility at 64 h, indicating the slow process of macromolecular hydration in untreated seeds. It is interesting to note that in both control and treated seeds, the third component of water with least mobility (bound water) appears when the seed reaches the critical water content of about 1.33-1.41 g g^{-1} DW. Exposure of *S. seabrana* seeds to microwave energies partly reduced hardseededness and increased germination percentage. The mode of action of microwave treatment can be attributed to improved permeability of seed coat membranes to water and its distribution into the seed during imbibition and to increased macromolecular hydration essential for the completion of germination process.

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