Indian J. Plant Physiol., Vol. 9, No. 1, (N.S.) pp. 47-53 (Jan.-March, 2004)

GERMINATION BEHAVIOUR OF *RAUWOLFIA SERPENTINA* BENTH. IN ASSOCIATION WITH VAM FUNGI

S. CHOUDHURY, J.B. BHANDARI AND K. GUPTA*

Botany Department, Burdwan University, Burdwan – 713104, West Bengal, India.

Received on 7 March, 2003, Revised on 19 Dec., 2003.

SUMMARY

Freshly harvested seeds of *Rauwolfia serpentina* benth. after sun drying failed to germinate in laboratory at 25ºC in light or in dark. Treatments like acid scarification with sulfuric acid, de-coating by sodium hypochlorite and alternate temperature alone or in combination also failed to cause germination. Imbibition percentage (weight basis), however, increased over control. Scanning Electron Microscopic studies of seed coat revealed that pretreatments were differentially effective in opening the hilum and suture region, the effectiveness being maximum in acid scarification. Fresh seeds showed 15% germination when they were associated with vesicular arbuscular mycorrhizal (VAM) fungi. Germination percentage increased when seeds were exposed to alternate temperature (35%), sodium hypochlorite (60%) and sulfuric acid (80%) pretreatment in association with VAM fungi. Initiation of germination also accelerated with these treatments. Scarification treatment increased leaching of electrolyte, soluble carbohydrate and amino acids, which probably act as chemical messenger, that assists in coordinating the formation of mycorrhiza.

***Key words*: Germination, *Rauwolfia serpentina*, VAM.**

INTRODUCTION

Rauwolfia serpentina Benth. is among the most important medicinal plants native to India. The roots of the plant have been used in the indigenous system of medicine from ancient times for the treatment of various ailments. Approximately 400-500 tons of roots are being exploited mainly from the forests in India (Husain 1993). Due to their over exploitation for commercial use, the plant has become rare in most of the accessible areas of its natural occurrence (Jain and Sastry 1980). A large number of important indole alkaloids have also been isolated from this plant like ajmalicine, ajmaline, reserpine, rescinamine, serpentine etc.

Wild plants of *R. serpentina* grows in shady moist or sometimes swampy areas. In cultivation trials, propagation is chiefly done through seeds. The rate of germination, however, is quite variable ranging from 10% to 74% in case of fully matured heavy seeds (Husain 1993). Germination rate of the seeds also differs under varying agro-climatic conditions. Direct sowing of the seeds in the field has not been found successful and hence seedlings are raised in nursery. The nurseries preferably located in shaded areas are kept moist through out the germination period to facilitate seed germination. So for successful propagation and biomass production, the basic need is to raise seedlings from healthy seed lots.

Contribution of mycorrhizae to growth, development and yield of plants is now an established fact. Their role, however, in controlling germination of hard-coated seeds have not yet been studied in commercial crops except in case of Australian Orchids (Wilkinson *et al.* 1989). In the

* Corresponding author

present study, an attempt has been made to study the effect of VAM and other treatments (which generally rupture coat-imposed barrier on germination) on germination behaviour of *R. serpentina.*

MATERIALS AND METHODS

Seeds of *R. serpentina* Benth. were collected form the experimental garden of the Botany Department, Burdwan University and sun-dried. They were stored in plastic bags at 10 ± 1ºC. The following experiments were conducted with the seeds.

Effect of alternate temperature treatment on coated seeds : For these pretreatments followings sets were made :

(i) Soaking of seeds in 10 ml of distilled water at 25ºC

(ii) Soaking at 60ºC for 30 minutes followed by 5ºC for 60 minutes

(iii) Soaking at 80ºC for 30 minutes followed by 5ºC for 60 minutes

(iv) Soaking at 80ºC for 30 minutes followed by 0ºC for 60 minutes

(v) Soaking at 80ºC for 30 minutes followed by 0ºC for 120 minutes.

The treated seeds were allowed to imbibe distilled water (DW) for 24h at 25ºC in the dark. After imbibition, the seeds were taken out, blotted dry and transferred to petridishes lined with moist Whatman No. 1 filter paper. All the seeds were allowed to germinate at 25ºC in light.

Effect of scarification treatments: Both coated and alternate temperature (80ºC for 30 minutes / 0ºC for 120 minutes) treated seeds were immersed in 10 ml of DW (control) or treated with either 10 ml of 36, 18 and 9N sulfuric acid for 30, 60, 90, 120 and 180 minutes respectively (sulfuric acid treatment) or 10 ml of 3% sodium hypochlorite solution for 1, 2 and 3 hours (sodium hypochlorite treatment). Seeds were washed after treatment in running tap water for 2h and allowed to germinate in the same way as in the former set.

Effect of VAM

For analyzing the effect of mycorrhiza, three categories of seeds were taken i.e., i. intact seeds, ii. decoated seeds (decoating done by treatments with 18 N sulfuric acid for 3 h or by 3% sodium hypochlorite for 3 h followed by washing in running water for 2h) and iii. seeds pretreated at 80ºC for 30 minutes followed by 0ºC for 120 minutes. The treated seeds were soaked in 10 ml of DW at 25ºC in the dark for 24 h. After imbibition, seeds were taken out, inoculated with soil/roots containing spores of mycorrhizal fungi and transferred to small earthen pots containing normal and sterilized garden soil and allowed to germinate at 25ºC in 16 h light and 8 h dark photoperiod. All sets of treatments contained four replicates, each of 25 seeds. Daily germination counts were made by recording number of seeds with 2 mm of radicle protruding through the testa. Total germination percentage (G) and days required for initiation of visible germination (TI) were analysed as recommended by Furutani *et al*. (1985).

Changes in the hylum, suture and external wall caused by different scarification treatments were observed under a Scanning Electron Microscope (SEM). Seeds were dried for 7 days in a desicator containing silica gel. Seeds were previously vaccum coated with gold using a IB-2-ion coater chamber. Electron microphotographs were prepared using a SEM (Hitachi S-530 at 15 KV).

Test for seed leaching

Seeds (250mg) of each treatment were undertaken in 6 replicates in 100 ml beakers containing 25 ml of double distilled deionized water and kept at 25ºC in the dark for 24h. The seeds were then removed and the pooled leachate was tested for conductivity of leachates, soluble carbohydrates and free amino acids. Electrical conductivity of the pooled leachates (25 ml) were determined by conductivity meter directly. The result was expressed in terms of µMhos $(25\ ml)^{-1}$. Soluble carbohydrate content was determined following the method of McCready *et al*. (1950) and expressed in terms of mg^{-1} g dry wt. Amino acids in the leachate was determined following the method of Moore and Stein (1948) and expressed in terms of mg^{-1} g dry wt.

For viability test, seeds were placed in 0.1% TTC (2, 3, 5 — triphenyl tetrazolium chloride) solution at 37ºC for 3 h. Reddish-pink colour of formazan in the embryonic axis denoted viability.

Data on germination parameters were collected and analysed statistically.

RESULTS AND DISCUSSION

Removal of cuticular layer of seeds by acid treatment or by sodium hypochlorite treatment increased the germination percentage of many seeds (Masuda and Konishi 1993, Choudhury and Gupta 1998). Treatments of sulfuric acid and sodium hypochlorite for different duration, different concentrations and different temperatures were effective in increasing percentage imbibition of *R. serpentina* (Table 1). Highest percentage of imbibition was obtained in alternate temperature treatment i.e. in 80ºC for 30 minutes/0ºC for 120 minutes. All the seeds remained viable (as evidenced from TTC test), but no visible germination was observed in any case. In a parallel set of treatments, when sulfuric acid and sodium hypochlorite with different concentrations and duration were given to the seeds receiving optimum alternate temperature treatment, it was observed that imbibition percentage (on weight basis) was increased when combination treatments were given (Table 2). Except long duration of concentrated sulfuric acid treatment all the seeds responded to TTC test indicating maintenance of viability. Chagtai *et al*. (1991) and Seal and Gupta (1999) reported that scarification of *Catharanthus roseus* and *Sida acuta* seeds with sulfuric acid retarded the germination and growth significantly in longer incubation period. Pullock and Toole (1961) reported that acid scarification increased seed germination by improving the seed coat permeability and removing the blockage of gaseous exchange. But such a seed coat effect does not appear to be the sole factor of germination of *R. serpentina*

Table 1. Effect of different scarification treatments on germination physiology of *R. serpentina* seeds.

Treatments	Duration of treatment (minutes)	Imbibition (%)	Germination (%)	Viability
Control		14.94	-	+
36 N H_2SO_4	60	52.13	-	+
36 N H_2SO_4	120	44.51	-	+
36 N H_2SO_4	180	31.52	-	+
18 N H_2SO_4	60	51.54	-	+
18 N H_2SO_4	120	57.99	-	+
18 N H_2SO_4	180	53.27	-	+
9 N H_2SO_4	60	39.17	-	+
9 N H_2SO_4	120	49.41	-	+
9 N H_2SO_4	180	60.94	-	+
3% sodium	60	29.82	-	+
hypochlorite	120	41.6	-	+
	180	51.92	-	+
Alternate	60ºC (30 min)/5ºC (60 min)	52.13	-	+
temperature	80ºC (30 min)/5ºC (60 min)	56.19	-	+
treatment	80ºC (30 min)/0ºC (60 min)	61.95	-	+
	80ºC (30 min)/0 ºC (120 min)	70.56	-	+
L.S.D. at 5%		11.289		

'-' denotes no visible germination

'+' denotes viability.

Table 2. Effect of different combination of scarification treatments on germination physiology of *R. serpentina* seeds.

Treatment	Duration of treatment (minutes)	Imbibition (%)	Germination (%)	Viability
Control		14.94	-	+
80ºC (30 min)/0ºC (120 min) [A]		70.56	-	+
A + 36 N H_2SO_4	30	73.2	-	+
A + 36 N H_2SO_4	60	59.98	-	*
A + 36 N H_2SO_4	90	-	-	-
A + 18 N H_2SO_4	30	84.36	-	+
A + 18 N H_2SO_4	60	61.89	-	+
A + 18 N H_2SO_4	90	52.57	-	+
A + 9 N H_2SO_4	30	75.29	-	+
A + 9 N H_2SO_4	60	81.21	-	+
A + 9 N H_2SO_4	90	87.82	-	+
A + 3% sodium hypochlorite	60	95.11	-	+
	120	104.67	-	+
	180	116.37	-	+
L.S.D. at 5%		23.57		

'-' denotes no visible germination

'*' denotes 50% viability

'+' denotes viability.

seeds because complete decoating actually caused loss of viability.

Because of the widespread association of VAM fungi with the agronomically important plants, it appears that VAM play a major role in sustained plant production in agriculture. In germination studies, however, the reports are very scanty. Both coated and decoated seeds of *R. serpentina* showed no germination in absence of mycorrhizal association in normal as well as sterilized garden soil (Table 3). The gradual increase in germination percentage from 15 to 80% was observed with different pretreatments in association with VAM. Highest germination (80%) was found in sulfuric acid (18 N for 3 h) pretreated seeds with VAM association. Wilkinson *et al*. (1989) established the role of bacteria and mycorrhizal tissues of terrestrial orchids in affecting the germination of orchid seeds. T_1 (days required for initiation of visible germination) was also reduced in sulfuric acid treatment followed by sodium hypochlorite and alternate temperature treatments. It appears that the formation of the first entry point is a critical stage in VAM development (Mosse and Hepper 1975) and that changes in the root exudation patterns and the hormonal balance in the plant are involved in the establishment and development of symbiosis (Barea 1986).

Scanning Electron Microscopy (SEM) of the different parts of the seed of *R. serpentina* like hilum, suture and external wall showed that different pretreatments like alternate temperature, sodium hypoclorite, sulfuric acid provided passages by breaking or degrading the above mentioned seed structures which may facilitate the connections between internal seed tissue and external media (Plate I & II). This effect was found to be drastic in sulfuric acid treatments where clear opening at the hilar regions along the suture was noticed (Plate II). Koske and Gemma (1992) established the possible communication between mycorrhizal fungi and plant roots prior to formation of mycorrhizae. It was observed that living roots release a wide variety of soluble, insoluble and volatile exudates, some of which act as chemical messengers that assist in coordinating the formation of mycorrhiza. In the earlier studies it was noted that VAM development was highly correlated with the amount of

Table 3. Effect of mycorrhizal association on germination physiology of *R. serpentina* seeds.

Treatment	Soil condition	Germination percentage (%)	Initiation of Germination (days)	Viability
	Normal	-	-	+
Control	Sterilized	-	-	+
	Mycorrhizal	15	32	+
Alternate temperature	Normal	-	-	+
[80ºC (30 min)/	Sterilized	-	-	+
0ºC (120 min)]	Mycorrhizal	35	30	+
Sulfuric acid	Normal	-	-	+
[18 N for 180 min]	Sterilized	-	-	+
	Mycorrhizal	80	21	+
Sodium hypochlorite	Normal	-	-	+
(3% for 180 min)	Sterilized	-	-	+
	Mycorrhizal	60	27	+
L.S.D. at 5%		13.85	1.52	-

'-' denotes no visible germination
'+' denotes viability.

Table 4. Effect of scarification treatments on electrolyte leakage, soluble carbohydrate and amino acid content in *R. serpentina* seeds.

Scarification treatment	Electrolyte (µmho) leakage ($25ml^{-1}$)	Soluble Carbohydrate content (mg g^{-1} dry wt.)	Free amino acid content (mg g^{-1} dry wt.)
Control	78.42	17.196	0.326
Alternate temperature Treatment [in 80ºC for 30 minutes /0ºC for 120 minutes]	91.9	21.661	0.564
Sulfuric acid [18 N for 180 min]	129	30.652	1.188
Sodium hypochlorite (3% for 180 min)	112	26.565	0.862
L.S.D. at 5%	8.294	1.652	0.057

the exudates (reducing sugar and amino acids) and it appears that some of these compounds served as carbon sources for the fungi (Dixon *et al*. 1988, Mosse 1988). In the present studies also leaching of electrolytes, soluble carbohydrate and amino acids were significantly increased in treatments. It was also observed that the decoating experiments like sulfuric acid and sodium hypochlorite pretreatments induced the excessive release of different leachates. The breakage of the seed coat by different scarification pretreatments probably enhance the imbibition percentage, leaching of electrolytes, sugars and amino acids but protrusion of root primordia occurs only through VAM association in causing germination. Possible causes of VAM effect on germination are being investigated.

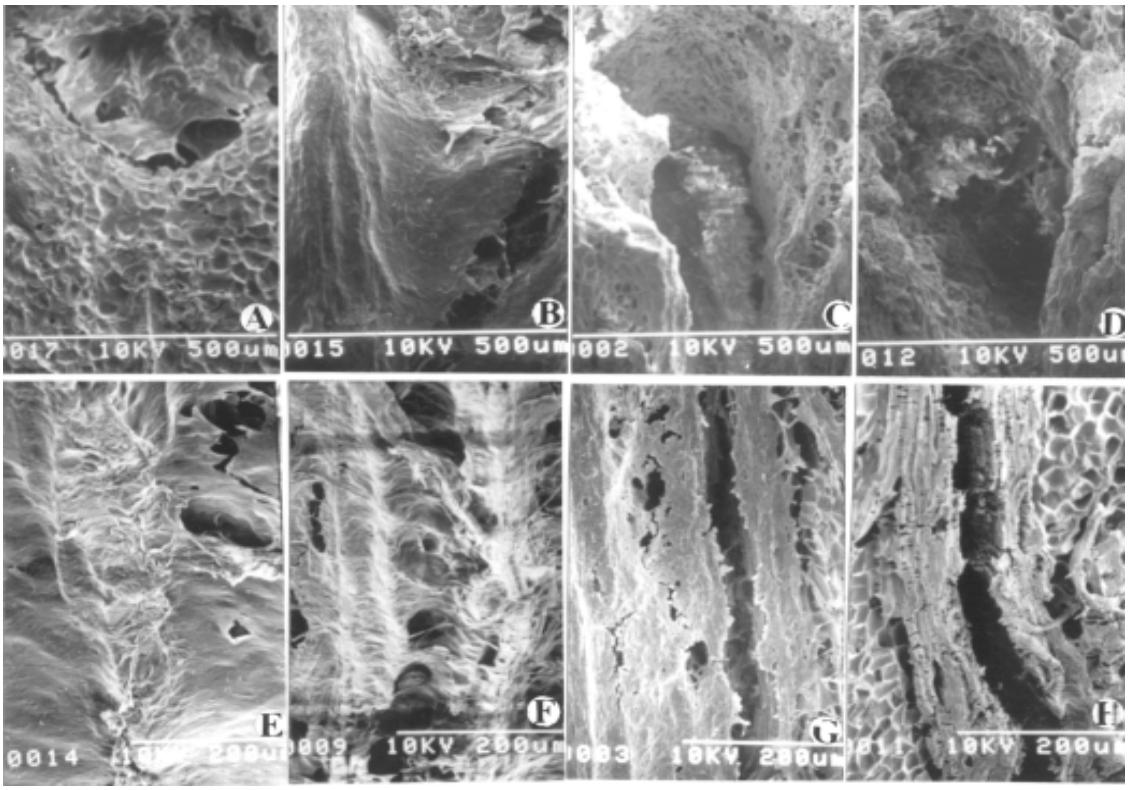

Fig. 1. Scanning Electron Micrographs of hilar region (A-D) and suture region (E-H) of *Rawolfia serpentina* seeds under water (control), alternate temperature (in 80ºC for 30 minutes /0ºC for 120 minutes), sodium hypochlorite (3% for 180 minutes) and sulfuric acid (18N for 180 minutes) respectively.

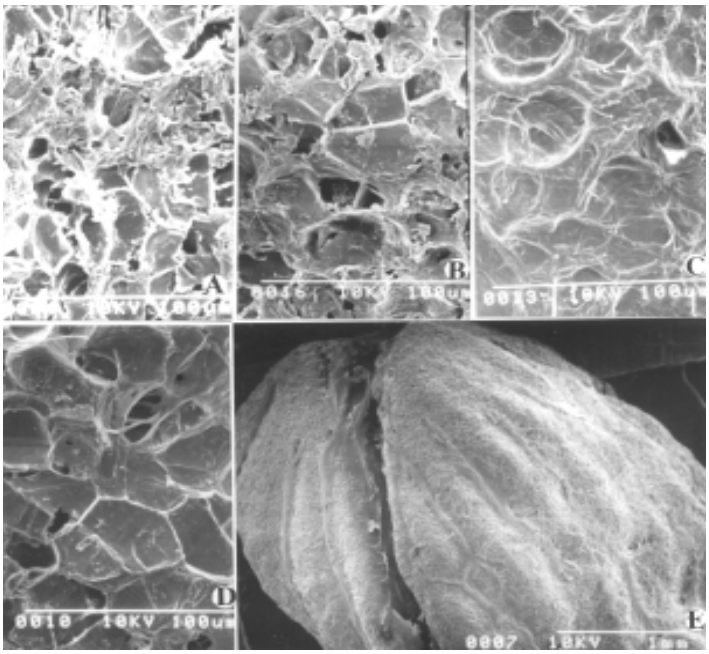

Fig. 2. Scanning Electron Micrographs of external wall region (A-D) of *Rawolfia serpentina* seeds under water (control), alternate temperature (in 80ºC for 30 minutes / 0ºC for 120 minutes), sodium hypochlorite (3% for 180 minutes) and sulfuric acid (18N for 180 minutes) respectively and whole seed (E) treated with 18N sulfuric acid for 180 minutes.

ACKNOWLEDGEMENTS

The authors are indebted to the CSIR, New Delhi, for financial assistance and to the University of Burdwan for providing necessary research facilities.

REFERENCES

Barea J.M. (1986). Importance of hormones and root exudates in mycorrhizal phenomena. In: V. Gianinazzi Pearson and S. Gianinazzi (eds.) Physiological and Genetical Aspects of Mycorrhizae, pp. 117-187. INRA, Paris.

Chaghtai, S.M., Ibrar, M. and Ali, Q. (1991). Some autecological observations on *Catharanthus roseus* (L.) G. Don. *Pak. J. Bot*. **23**: 249-256.

Choudhury, S. and Gupta, K. (1998). Studies on the germination mechanism of *Catharanthus roseus* (L.) G. Don cv. *alba* seeds: Effects of promoters and pH. *Seed Sci. & Tech.* **26**: 719-732.

Dixon R.K. Garret, H.E. and Cox, G.S. (1988). Carbohydrate relationship of *Citrus jambhiri* inoculated with *Glomus fasciculatum. J. Am. Soc. Hort. Sci*. **113**: 239-242.

Furutani, S.C., Zandstra, B.H. and Price, H.C. (1985). Low temperature germination of celery seeds for fluid drilling. *J. Am. Soc. II ort. Sci*. **110**: 153-156.

Husain, A. (1993). Medicinal Plants and Their Cultivation. Central Institute of Medicinal and Aromatic Plants, Lucknow.

Jain, S.K. and Sastry, A.R.K. (1980). Threatened Plants of India — A state of the art report. Botanical Survey of India. Calcutta.

Koske, R.E. and Gemma, J.N. (1992). Fungal reactions to plants prior to mycorrhiza formation. In: M.J. Allen (eds.), Mycorrhizal Functioning, An Integrative Plant-Fungal Process, pp. 3-36. Chapman & Hall, London.

Masuda, M. and Konishi, K. (1993). Improvement of high temperature germination of spinach seeds with acid scarification and priming with polyethylene glycol 6000. *J. Jap. Soc. Hort. Sci*. **62**: 419-429.

McCready, R.M., Gruggolz, J., Silviera, V. and Owens, H.S. (1950). Determination of starch and amylase in vegetables. *Anal. Chem*. **22**: 1156-1158.

Moore, S. and Stein, W.W. (1948). Photometric ninhydrin method for use in the chromatography of amino acids. *J. Biol. Chem*. **176**: 367-368.

Mosse, B. (1988). Some studies relating to "independent" growth of vesicular-arbuscular endophytes. *Can. J. Bot*. **66**: 2533-2540.

Mosse, B. and Hepper, C. (1975). Vesicular-arbuscular mycorrhizal infections in root organ culture. *Physiol. Plant Pathol*. **5**: 215-223.

Pullock, M.B. and Toole, V.K. (1961). Seeds. In The Year Book of Agriculture. U.S.D.A. Washington. DC.

Seal, S. and Gupta, K. (1999). Effect of temperature, concentrated H_2SO_4 and nonpolar solvents on removal of coat imposed dormancy in *Sida acuta* Burm. f. and *S. rhombifolia L. Indian J. Plant Physiol*. **4**: 175-178.

Wilkinson, K.G., Dixon, K.W. and Sivasithamparam, K. (1989). Interaction of soil bacteria, mycorrhizal fungi and orchid seed in relation of soil germination of Australian orchids. *New Phytol*. **112**: 429-435.

Zar, J.H. (1974). Biostatistical Analysis. Prentice-Hall Incorporation. Englewood Cliffs, New Jersey, London.