research article | *International Journal of Plant Sciences,* (July, 2010) Vol. 5 Issue 2 : 664-668

Biochemical aspects of desiccation induced viability loss in *Myristica malabarica* Lam. seeds

K.P. BABU, C. ANILKUMAR AND NABEESA SALIM

Accepted : June, 2010

SUMMARY

Desiccation sensitivity of recalcitrant seeds of *Myristica malabarica* was studied by exposing freshly collected mature seeds to room temperature 28± 2°C, and 60%RH.)Moisture content and germination rate were reduced uniformly and viability (72%) was retained up to 6 days when the moisture content was reduced to one half. Electrolyte leakage and lipid peroxidation showed linear increase while formozan intensity was reduced gradually until the loss of viability. Peroxidase and polyphenoloxidase were more active up to 4th day of desiccation compared to the control whereas, drastic reduction in the activity of these enzymes was observed coinciding with loss of viability. Even though *Myristica malabarica* seeds contained only 27% moisture content and were considered as moderately recalcitrant because these seeds were highly sensitive to desiccation and the loss of viability begans after one day and ends within 7-8days. The desiccation sensitivity appeared to be due to manifold electrolyte leakage and lipid peroxidation and comparatively reduced enzymatic protection expressed as peroxidase and polyphenol oxidase against free radicals formed due to desiccation stress.

Key words : *Myristica malabarica*, Viability loss, Desication

Desiccation sensitivity is the well documented characteristic of recalcitrant seeds (Lin and Chen, 1995; Farrent *et al*., 1996; Finch-Savage, 1996; Pammenter and Berjack, 1999). Viability loss in recalcitrant seeds is the synergistic effect of a large number of metabolic processes. These include mechanical stresses upon the removal of water which cause structural changes at sub cellular (Drew *et al*., 2002; Hilhorst *et al*., 2004) cellular (Kozeko and Troyan, 2000) and tissues levels (Liang and Sun, 2000). Alterations in the membrane structural integrity and function due to desiccation are well documented and reflected by the increased leakage of ions, sugars and proteins (Chaithanya and Naithani, 1994; Finch-Savage *et al*.,1996). The breakdown of metabolic co-ordination in cells may initiate uncontrolled free radical attack and decrease enzymic and non-enzymic protein protection against such oxidative damages (Leprince *et al*., 1990, Hendry *et al*., 1992; Hendry, 1993 Pammenter *et al*., 1994, Pammenter and Berjack, 1999). An important process of viability loss during desiccation of recalcitrant seeds is the formation of free radicals and highly reactive oxygen species which are synthesised as a result of impaired oxidative metabolism (Chaithanya and Naithani, 1994; Come and Corbineau, 1996; Leprince *et al*., 1999, Pammenter and Berjack,1999;Greggains *et al*., 2001). A number of protective enzymes like peroxidase, polyphenol oxidases and super oxide dismutase against the highly reactive free radicals have been reported in recalcitrant seeds (Chaithanya and Naithani, 1994; Finch-Savage *et al*., 1996).

Myristica malabarica is an endemic tree of Western Ghats in peninsular India, a major floristic component in *Myristica swamps*. The riparian climate is suited for the easy germination of the recalcitrant seeds, of *Myristica malabarica* Anilkumar *et al*. (2002) reported the desiccation and chilling sensitivity of moderately recalcitrant seeds of this taxon. The present study highlights the effects of desiccation on metabolism, leading to loss of viability. The investigation includes the analysis of moisture content, germinability, measurements of electrical conductivity, lipid peroxiation, reduction of tetrazolium chloride, and assay of scavenging enzymes like peroxidase and polyphenol oxidase during desiccation.

MATERIALS AND METHODS

Fully mature seeds of *Myristica malabarica* were collected from *Myristica swamps* of evergreen forest of Kallar in Southern Western Ghats, Kerala, India. Harvesting maturity of seeds were inferred when some

Correspondence to:
K.P. BABU, Division of Seed Bank, Tropical Botanical Garden and Research Institute, Palode, THIRUVANANTHAPURAM (KERALA) INDIA
Authors' affiliations:
C. ANIL KUMAR, Division of Seed Bank, Tropical Botanical Garden and Research Institute, Palode, THIRUVANANTHAPURAM (KERALA) INDIA
NABEESA SALIM, Division of Physiology and Biochemistry, Department of Botany, Calicut University, MALLAPURAM (KERALA) INDIA

of the fruits started splitting open through their longitudinal fruit valves. After hand harvesting fruit valves were separated manually and then surface sterilized with 0.1%$HgCl_2$ and washed four times with distilled water. Seed lots consisting of 100 seeds each in triplicates were kept open at laboratory conditions (28±2^{0}C,60%RH.) for desiccation and samples for the following biochemical studies were collected at 5 intervals namely 1st 2nd, 4th, 6th. 8th days of desiccation. Fresh seeds served as control all experiments were repeated five times for statistical analysis of data.

Germination studies and moisture content:

Three replicates of twenty seeds each from all samples (control treatments) were kept in acid free paper towels and incubated in a seed germinator (90%RH and 30^{0}C). The seeds were considered germinated when the radicle protruded from the testa. Samples for all analyses were taken from randomised seed lots. Moisture content was calculated following ISTA rules (1985) by drying at 103^{0}C for 16 hours and expressed as percentage moisture content on fresh weight basis.

Conductivity measurements:

One seed each in five replicates from all seed lots kept for desiccation was incubated in 50ml distilled water for overnight. Conductivity of the leachate was measured using a conductivity meter (systronics model No.306).

Lipid peroxidation:

Lipid peroxidation was measured as the concentration of thiobarbituric acid reactive substance (TBRS) equated with malondialhide (MDA) according to Heath and Packer (1968). Known weight of issue was homogenised in distilled water and 5% thiobarbituric acid was added and incubated in boiling water bath for 30 minutes. After cooling to 0^{0}C, the supernatant was collected and colour was measured using spectrophotometer (Schimadzn) at 540 nm and expressed as A 540/g weight of the tissue.

Tetrazolium chloride reduction activity:

Known quantities of the seeds were incubated in 1, 2, 3, 5 % Triphenyl tetrazolium chloride solution in dark for two hours. After three washes in distilled water the staining pattern was observed. The tissue was chopped and the formazan formed was extracted with acetone. Optical density of the solution was measured after centrifugation and expressed as A 540/g fresh weight (Kittock and Law, 1968).

Extraction of enzymes:

Enzymes were extracted in cold 0.01 M phosphate buffer (pH 7.2) containing 1.5% poly vinyl poly pyrolidone (PVPP). The homogenate was centrifuged using a refrigerated centrifuge at 5000g for 20 minutes and the supematant was precipitated in cold acetone and kept in an ice bath for 30 minutes. The pellet was washed with acetone twice and after the complete evaporation of acetone the pellet was resuspended in phosphate buffer. After centrifugation the supermatant was used as enzyme source for both peroxidase and polyphenol oxidase assay. All enzymatic studies were conducted at 4^{0}C.

Peroxidase assay (EC 1.11.1.7):

Peroxidase activity was measured according to Chance and Machly (1955). Three ml of the assay mixture contained 0.1 M phosphate buffer (pH7.2), 12mm guaiacol and enzyme extract. The reaction was triggered by adding 0.1 ml of 13 mM H_2O_2 to the mixture. The change in absorbance was recorded immediately at 470 nm using spectrophotometer (Schimadzu) model. The enzyme activity was calculated and expressed as change in absorbance per min/fresh weight tissue

Poly phenol oxidase assay (EC 1.14.18.1):

The method of Yamaugchi *et al*. (1970) was followed to estimate polyphenol oxidase activity. The assay mixture contained 0.1 catechol, 0.1 M praline in 0.1M phosphate buffer (pH.6.8) and 0.5 ml enzyme. The kinetics of the reaction was immediately recorded at 525nm using spectrophotometer. The activity was expressed as change in absorbance/min/g.fresh weight.

RESULTS AND DISCUSSION

Fresh seeds of *Myristica malabarica* contained 0.277g g^{-1} moisture and registered 100% germination. Exposure of seeds to open laboratory conditions (28+2^{0}C/ 60% RH) recorded a decrease in moisture content and germination (Table 1). After 6 days of desiccation of the seed moisture content and germination was reduced to 0.144g g^{-1} and 72%, respectively. Further desiccation of seeds resulted in the reduction of moisture content and a marked effect on the reduction of seed viability. Exposure for a prolonged period of 8 days, the seed moisture content was lowered to 0.102gg^{-1} and germination was only 20%.

When fresh seeds and those desiccated for 48 hours were incubated in tetrazolium chloride solution both the embryonic axis and cotyledons were deep red. Absorbance of formazan extract was maximum for the control seeds. But when the seed moisture content was reduced to 0.144 g g^{-1} on the 6th day the corresponding

Table 1 : Effect of desiccation on *Myristica malabarica* seeds

Period of desiccation days	Moisture content %± SE	Germination% ± SE
0	27.7 ± 1.08	100
1	23.5 ± 1.1	92 ± 2
2	21.7 ± 1.12	88 ± 2.5
4	19.1 ± 1.01	86 ± 3.5
6	14.4 ± 0.87	72 ± 2.4
7	12.0 ± 0.82	52 ± 2.1
8	10.23 ± 0.68	20 ± 1.6

absorbance was very low (Table 2).

The electrolyte leakage represented as conductivity measurements showed linear increase in solute leakage during desiccation and after 8 days the conductivity was increased almost tenfold (Table 2). The production of thiocarbituric acid reactive substance (TBRS) as a result of lipid peroxidation was increased proportional to the period of desiccation (Table 2).

Table 2 : Response of desiccation in *Myristica malabarica* seeds towards desiccation

Period of desiccation days	Conductivity	Formazane concentration OD	Lipid peroxidation OD
0	12.5 ± 0.8	0.375	0.121
1	32.3 ± 1.1	0.317	0.162
2	41.8 ± 1.3	0.205	0.176
4	63.1 ± 2.3	0.172	0.217
6	115 ± 3.6	0.062	0.227
7	119 ± 4	0.02	0.236
8	121 ± 4.2	0.002	0.254

Both the anti-oxidant enzymes (Peroxidase and Poly phenol oxidase) showed increase in early days of desiccation. The peroxidase activity was maximum on 4th day of desiccation and afterwards significant reduction was occurred. Poly phenol oxidase activity registered the highest value after 2 days and gradually decreased up to 8 days propotemol to the period of desiccation.

The swampy riparian climate provides an optimum temperature and relative humidity for the germination of recalcitrant tree seeds of *Myristica malabarica* in Western Ghats of Kerala, India. Even though the seed coat is thick hard the seeds are highly sensitive to desiccation fresh seeds show cent per cent germination, and desiccation for one day reslts in 4% reduction in moisture content and slight loss of viability. (Anil Kumar *et al* ., 2002) Seeds of *Myristica malabarica* withstand 48% (MC,14.4% reduction in moisture content up to 6 days of storage (Table 1) and the corresponding germination is 69%. Direct correlation between moisture content and germination percentage of *M.malabarica* seed has already been reported (Anilkumar *et al.*, 2002).

Tetrazolium chloride tests using formazan optical density values obtained during desiccation period showed very low dehy drogenases action to due to reduced respiratory rate that this parameter is agreable with the correlation between desiccation and seed liability (Table 2).

The linear increase in specific conductance of the leachate may be due to the desiccation induced membrane damage of *Myristica* seeds and this observation is in conformity with the seed behaviour of *Machilus thunbergii* (*Lin and Chen*, 1995) and *Theobroma cacao* (Li and Sun, 1999) . The ionic leakage is caused by physical broaching of the cell membrance by the inrush of water during rehydration (Zheng, 1991). Irreversible solute leakage related to loss of viability in recalcitrant seeds has been reported by many authors (Berjack *et al*., 1989, Fu *et al*.,1990).

Moisture content and water potential are not directly related each other in reelalcitrant seeds because water potential range varies with seed composition which is likely to be differed between seed samples (Pammenter and Berjak, 1999) Moisture content of *M. malabarica* seeds desiccated for 6 days was reduced to one half (Table) and concomitantly viability fallen below 70% and the corresponding electrolyte conductivity showed ten fold increase compared to the control seeds. Although in the present investigation, water potential was not taken as a parameter, manifold increase in the electrolyte conductivity resulted in a significant debction of water potential leading to desiccation stress and loss of seed viability.

Free radical generation as a consequence uncoordinated metabolism is a major injurious factor during dehydration of recalcitrant seed (Come and Corbineau, 1996. According to Leprince *et al*. (1993) one of the protective systems involved in desiccation tolerance is the ability to prevent the free radical attack. During desiccation, increased respiration and cipia peroxidation produce free radicals in recalcitrant seeds and for combating with these free radicals seeds are endowed with antioxidant molecules (Finch – Savage *et al*., 1994) and scarenging engages (Leprince *et al*., 1993).

Enhanced activity of peroxidase and polyphenol oxidase in the seeds of *Guilfoylia monostylis* (NKang, 2001) and *Telfairia occidentalis* (NKang *et al*., 2003) are related to desiccation tolerance. As a protective mechanism against desiccation, increased activity of both peroxidase and polyhenol oxidase was observed in *M.*

Table 3 : Effect of desiccation on peroxidase and polyphenol oxidase activity in *Myristica malabarica*

Period of desiccation days	Peroxidase (A470/min)	Polyphenol oxidase (A 525/min)
0	0.234 ±0.03	0.410 ±0.02
2	0.240 ± 0.02	0.571 ± 0.03
4	0.257 ± 0.01	0.469 ± 0.03
6	0.108 ± 0.01	0.286 ± 0.01
7	0.103 ± 0.02	0.167 ± 0.02

malabarica seeds. Activity of both the enzymes were maximum during 2-4 days of desiccation. The detrimental effect of free radicals during desiccation of recalcitrant seeds and the protective role against such damages by enzymatic process have been reported in may seeds and this defence mechanism varies among species (Hendry *et al*., 1992; Pammenter *et al.*,1994; Li and Sun, 1999). In *M. malabarica* peroxidases and polyphenol oxidases were active in fresh seeds and this same trend was maintained rather increased only slightly as step to a days of desiccation followed by drastic reduction in the activity, which coincided with loss of seed viability. The rapid browning of desiccated seeds on exposure to air data not included is due to the oxidation of various types of phenolics involving the activity of poly phenol oxidases leading to the loss of viability in *M. malabarica* seeds. Most plausible reason for loss of viability in of *M.malabarica* seeds during desiccation may be inefficient functioning of antioxidant systems resulting in uncontrolled accumulation of free radicals through peroxidation of membrane lipids as confirmed by increased rate of lipid peroxidation (Table 2) whereas insignificant reduction of germination rate coincide with enhanced activity of polyphenol oxidases and proxidases during 2-4 on 2nd day of desiccation. But after 4th day, these enzyme activities were drastically reduced and hence the seeds were not protected from desiccation stress. The overall metabolic changes during desiccation of *M. malabarica* seeds for a period of six days was the reduction of moisture content activity of poly phenol oxidase and peroxidase to one half, ten fold increase in electrolyte conductivity and doubling of lipid peroxidation rate. Even though the activity of the antioxidant enzymes is found to be effective way to avoid the detrimental effect of desiccation, the oxidative stress expressed as lipid peroxidation and resultant electrolyte leakage exceeded the antioxidant capacity of these enzymes and resultant short life span in the recalcitrant seeds of *M. malabarica*.

REFERENCES

Anilkumar, C., Babu, K.P. and Krishnan, P.N. (2002). Seed storage and viability of *Myristica Malabarica* Lam– an endemic species of Southern Western Ghats (India).

Berjack, P., Farrant, J.M. and Pammenter,N.W. (1989). The basis of recalcitrant seed behaviour. In:R.B.Taylorson.(Ed.) *Recent advances in the development and germination of seeds*. Plenum Press, New York 89-108 pp..

Chaithanya and Naithani, S.C. (1994) Role of superoxide, lipid peroxidation and super oxide dismutase in membrane perturbation during loss of viability in seeds of *Shorea robusta* Gaertn. *New Phytol.*, **126**:623-627.

Chance, B. and Machly, A.C. (1955). Assay of cartalase and peroxidase. *Methods in Enzymology*, **2** : 764 – 775.

Come, D. and Corbineau, F. (1996). Metabolic damages related to desiccation sensitivity. In:A.S. Ouedraogo, K.Poulsen, and F. Stubsgaard.(Eds.), *Intermediate/recalcitrant Tropical Forest /Tree* seeds.IPGRI Rom.

Drew, P.J. Pammenter, N.W. and Berjak, P. (2000). 'Subimbibed' storage is not an option for extending longevity of recalcitrant seeds of the tropical species, *Trichila dregena. Seed Sci.Res.*, **10**:355-365.

Farrant, J.M., Pammenter, N.W., Berjak,P., Farnsworth, E.J. and Vertucci, C.W.(1996). Presence of dehydrin like proteins and levels of abscisic acid in recalcitrant (desiccation sensitive). Seeds may be related to habitat. *Seed Sci. Res.*, **6** : 175-182.

Finch Savage, W.E. (1996). The role of developmental studies in research on recalcitrant and intermediate seeds. In A.S Ouedraogo.K. Poulsen And F.Stubsgaard (Eds). *Ingtermediate/Recalcitrant tropical forest tree Seeds.* IPGRL Rome 83-97pp.

Finch-Savage, W.F., Blake, P.S. and Clay, H.A. (1996). Desiccation stress sin recalcitrant *Quereus robur* L. Seeds. Results in lipid peroxidation and increased sythesis of Jasmontes and abscisic acid. *J.Expt. Bot.*, **47**:661-667.

Finch-Savage, W.E., Hendry, G.A.F. and Atherton, N.M.(1994) Free radical activity and loss of viability during drying of desiccation-sensitive tree seeds. Proceedings of RoyaL Society of Edinburgh, 102 B, 257-260.

Fu.J.R. Zhang, B.Z, Wang, X.P. Qiao. Y.Z., and Huang, X.L. (1990). Physiological studies on desiccation, wet storage and cryopreservation of recalcitrant seeds of three trait species and their excised embryonic axis. *Seed Sci.Technol.*, **18**:743-754.

Greggains, V., Finch-savage, W.E., Atherton, N.M. and Berjack, P. (2001). Viability loss and tree radical processes during desiccation of recalcitrant *Avicennia marina* seeds. *Seed Sci. Res.*, **11**:235-242.

Heath, R.L and Packer, L. (1968). Peroxidation. *Arch. Biochem. Biophysics*, **125**:189-198.

Hendry, G.A.F (1993). Oxygen, free radical processes and seed longevity. *Seed Sci. Res*., **3**:141-153.

Hendry, G.A.F, Rinch-savage, W.E., Thorpe, P.C., Atherton, N.M, Buckland, S.M., Nilson, K.A. and Seel, W.A. (1992). Free radical processes and loss of viability during desiccation I the recalcitrant species *Quercus robur* L. *New Phytol.*, **122**:273-279.

Hilhorst, H.W.,Faria, J.M.R., and Van Lammerea, A.A.M. (2004). Desiccation *Senility* and cell cycle aspects in seeds of *Inga vera* sub sp.*Affinis. Seed Sci. Res.*,**14** :165-178.

ISTA (1985). International Rules for seed testing. *Seed Sci. Technol.*, **13** : 338-341.

Kittock, D.L. and Law, A.G (1968). Relationship of Seedling vigour, respiration and tetrazolium reduction by germinating wheat seeds, *Agron. J.*, **60**:286-288.

Kozeko, L.E and Troyan,V.M. (2000). The relationship between the mitotic activity and moisture content of recalcitrant seeds of *Acer saccharinum* L., during mutation, Post mutation drying and germination seed. *Sci. Res.*, **10** : 225-232.

Leprince, O., Buitink, J. and Hoekstra, F.A. (1999). Axes and cotyledons of recalcitrant seeds of *Castanea sativa* Mill. Exhibit contrasting responses ofrespiration to drying in relation to desiccation sensitivity. *J. Exp. Bot.*, **50**:1515- 1524.

Leprince, O., Deltour,R., Thorpe, P.C., Atherton, N.M. and Hendry, G.A.F. (1990). The role of tree radicals and radical processing systems in loss of desiccation tolerance in geminating maize (*Zea mays*). *New Phytol.*, **116** : 573-580.

Liang, Y. and Sun, W.Q. (2000). Desiccating tolerance of recalcitrant *Theobroma cacaco* embryonic axis. The optimal drying rate and its physiologiocal basis. *J. Exp. Bot.*, **51** : 1911-1919.

Lin,T.P. and Chen, M.H. (1995). Biochemical characteristics associated with the development of the desiccation – sensitive seeds of *Machilus thunbergii* Sieb. & Zucc. *Ann. Bot.*, **76** : 381 – 387.

Li, C. and Sun, W.Q. (1999). Desiccation sensitivity and activities of free radical scavenging enzymes in recalcitrant *Theobroma cacao* seeds. *Seed Sci. Res.*, **9** : 209-217.

NKang, A. (2001) Effect of Cyanide pretreatment on the activities of polyphenol oxidase and peroxidase in the seeds of *Gnilfoyla monostylis*. *Seed Sci. Technol.*, **29**: 557-565.

NKang, A. Omokara, D. Egbe, A. and Amanke, G. (2003). Variations in fatty acid propotions during desiccation of *Telfairia occidentalis* seeds harvested at physiological and agronomic maturity. *Afr. J. Biotechnol.*, **2** : b33-39.

Pammenter, N.W. and Berjak, P. (1999). A review of recalcitrant seed physiology in relation to desiccation – tolerance mechaniss. *Seed Sci.Res.*, **9** :13-37.

Pammenter, N.W., Berjak,P., Farrant, J.M., Smith, M.T. and Ross, G. (1994). Why dostored, hydrated recalcitrant seeds die? *Seed Sci.Res.*, **4**:187-191.

Yamaguchi, M., Hwang, P.M. and compbell, J.D. (1970). Latent O-diphenol oxidase in mushrooms (*Agaricus bisporus*). *Can. J. Biochem.*, **48** : 198-2002.

Zheng, G.H. (1991). Physiological biochemical and ultrastructural aspects of garden. imbibitional chilling injury in seeds. *Seeds Sci.Res.*, **1** :125-134.

****
*****