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RESEARCH ARTICLE

Desiccation induced physiological and biochemical changes of *Gymnacranthera canarica* (King) Warb. seeds in the Myristica swamp forests, Southern Western Ghats, India

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Abstract

Gymnacranthera canarica (King) Warb. is an endemic tree species that dominates the Myristica swamp ecosystem of southern Western Ghats. This tropical tree species has become more threatened due to limited natural seed germination and habitat loss. Mature seeds were collected from the myristica swamp ecosystem subjected to desiccation study. This research evaluated the physiological (moisture content, tetrazolium reduction, lipid peroxidation, electrolyte leakage) and biochemical response of seeds during different desiccation treatments. Results showed that *G. canarica* seeds are highly sensitive to desiccation and total viability loss was seen within 15 days following harvest indicating the active seed metabolism of mature seeds showing absence of metabolic arrest. Desiccation enhanced malondialdehyde and electrolyte leakage while reducing formazan formation. Seed desiccation increases protease activity, which peaks when viability is lost. Desiccation reduced the quantity of phenol and starch, whereas proline, fat, sucrose and total soluble carbohydrates increased. The early viability loss in *G. canarica* seeds could be due to loss of membrane integrity, which was linked to ROS formation and associated lipid peroxidation products indicating seeds are truly recalcitrant.

Keywords

Gymnacranthera canarica, desiccation, MDA content, recalcitrance, moisture content, viability loss

Introduction

Myristicaceae family has a pantropical range distribution, with *Gymnacranthera canarica* (King) Warb. being endemic to the Myristica wetland forests of the Southern Western Ghats (1). Narrow distribution and recruitment failure due to high fruit or seed predation rate characterize the species niche specialization. This species has deep stilt roots, and the marsh floor is covered in looped knee roots, which provide excellent ecological services. The fruit aril is frequently used as wild nutmeg (2). Concerned about seeds as the primary propagation agent, researchers discovered that loss of viability due to desiccation was linked to a number of parameters, including oxidative stress caused by the generation of reactive oxygen species (ROS). Seeds were defined by Roberts on the basis of their storage behavior into orthodox and recalcitrant (4). Desiccation tolerance refers to a seed's ability to withstand drying and preserve physiological functions. ROS produced as a result of desiccation damage biological macromolecules such as proteins, lipids,

and nucleic acids (5) and desiccation can also trigger a potentially harmful autocatalytic lipid peroxidation process (6). Seed's ability to resist dryness and low temperatures while being viable for lengthy periods of time could be linked to their ability to eliminate ROS (7). Although substantial sucrose content was found in both tolerant and intolerant stages of cauliflower seeds, sucrose and raffinose play a key role in desiccation tolerance of orthodox seeds (8, 9). Carbohydrates are unlikely to be the only element affecting desiccation tolerance in *Arabiopsis thaliana* (L.) seeds (10). Seed protein in *Crotalaria retusa* seeds may be negatively affected during dehydration and desiccation, with processes such protein breakdown and alterations in memberane phospholipids leading to a loss of integrity (11). Propagation of *G. canarica* sp. from seed is really challenging and the natural regeneration was very poor and highly erratic. However, there is no information on *G. canarica* seed's ability to regenerate and the findings provided here are part of a wider study that aims to understand phenology, seed physiology, germination pattern and seedling establishment in order to determine the source of species scarcity in nature.

The objectives of current work were to understand the physiological and biochemical mechanisms of *G. canarica* seeds in relation to various levels of desiccation sensitivity.

Materials and Methods

Seed collection and preparation

Mature seeds of *G. canarica* were gathered in polythene bags from Myristica swamps in Kulathupuzha reserve forests of the Southern Western Ghats, Kerala, India: latitude 84'5N, longitude 77'10 E and altitude 15'5 MSL. The desiccation study used seed samples that were free of physical damage or insect infection. Thousands of seeds were completely washed with running water, dried on the surface, and dispersed in plastic trays for open desiccation at room temperature (28±2 °C, 60% RH). Every 24 hrs, seeds were randomly chosen to undertake various assays against freshly harvested seeds used as a control.

Moisture content determination

The moisture content of seeds and embryos in fresh and desiccated phases was determined using a low constant temperature hot air oven technique at 103 °C for 17 hrs (12). On a fresh weight basis, the following data from 6 replicates were calculated:

MC (%) = (fresh weight – dry weight/ fresh weight)×100

Germination

According to the International Seed Testing Association, germination experiments were conducted with 5 replicates of 10 fresh and desiccated seeds (control) at 24 hr intervals. These seeds were allowed to sprout in plastic bags filled with vermiculate soil at 27-28 °C until radicles developed (at least 5 mm). Germination percentage (GP) and speed of germination (SPG) were calculated, as well as peak value (PV), mean daily germination (MDG) and germination value (GV) (13). The germination test was performed for 55 days; beyond that time, the seeds became infected with fungus and were completely destroyed. After 90 days of germination, 10 seedlings were carefully taken from the soil by placing them in flat pans of water, where radicles could be easily detached from soil particles. The plumule and radicle lengths of each seedling were then measured. The formula for calculating seedling vigour index was used (14),

Vigour index (VI) = Germination (%) × Seedling length (mm)

Monitoring of electrolyte leakage

The EC Testr 11+ Multi range conductivity meter was used to measure electrolyte leakage in six replicates of seeds (15), each with 4 seeds, after they were immersed in 50 ml of deionized water for 24 hrs and the results were expressed in µs/cm/g.

Assessment of tissue viability

G. canarica seeds were soaked in deionized water overnight before being longitudinally cut and immersed in a 1 % (w/v) solution of 2, 3, 5-triphenyl tetrazolium chloride and incubated in the dark for 12 hrs at ambient temperature. The red-colored formazan that resulted was visually seen before being removed separately with acetone. Using a Schimadzu UV–VIS spectrophotometer, the absorbance of extracted formazan was measured at 520 nm and expressed as A520g-1 Fwt. A blank control sample was run through all of the processes but without TTC staining for each seed sample (16).

Total lipid content

One gram of weighted samples was homogenized in a combination of chloroform and methanol (2:1 v/v), stored overnight at room temperature in the dark, then centrifuged at 5000 rpm for 30 minutes following incubation. The clear lower layer of chloroform containing all lipids was carefully collected, evaporated and the amount of lipid calculated gravimetrically out of the 3 levels (17).

Lipid peroxidation rate

Desiccating seeds' lipid peroxidation was measured as 2-thiobarbituric acid (TBA) reactive metabolites, primarily malondialdehyde (MDA) (18). One gram of fresh seeds was extracted in two milliliters of 0.25 % TBA prepared in 10% TCA. The extract was heated to 95 °C for 30 min before being cooled immediately in ice. The absorbance of the supernatant was measured at 532 nm after centrifugation at 10000 g for 10 min. The absorbance value taken at 600 nm was subtracted for non-specific turbidity correction. Using an extinction coefficient of 155 mMcm-1, the degree of lipid peroxidation was represented as n mol of MDA produced.

Proline and total free amino acid determination

One gram of seed tissue was homogenized with 1 ml of cooled 3 % sulfosalicylic acid solution to determine the content of proline in desiccating seeds of *G. canarica*. At 4–8 °C, the homogenate was centrifuged at 12000 g for 2 min. Each sample was produced in an eppendorf tube with 0.2 ml acid ninhydrin, 0.2 ml 96 % acetic acid and

0.1 ml 3 % sulfosalicylic acid. Each homogenate's supernatant (0.1 ml) was put to glass tubes. After cooling, 1 ml of toluene was added to each tube after incubation at 96 °C for 1 hr in a hot water bath. At 520 nm, the pink red top phase showed absorbance. To determine the proline concentration in each sample, a standard curve for proline in the range of 0.01 mM to 1.5 mM was built using toluene as the blank (19). The ninhydrin technique was used to determine the total free amino acid content of desiccating seeds (20).

Determination of total phenol and total protein

Using the Folin-ciocalteau's reagent, total phenols were measured spectrophotometrically (Sigma). 1 gram of fresh sample from each desiccation step was homogenized and extracted 3 times using acidified methanol by centrifugation (12000*g) for 10 min at 4 °C and volume of combined supernatants were measured. After 3 min, 1 ml of saturated sodium carbonate solution was added and the mixture was built up to 10 ml with aliquots of extracts diluted to 7 ml and 0.5 ml of Folin–ciocalteau's reagent. The absorbance was measured at 725 nm after the reaction had been left for 1 hr. The total phenol content was reported as mg/g dwt SE using a standard curve made with quercetin (21). Using Bovine Serum Albumin as a standard, soluble proteins were quantified using the Lowry technique (22).

Sugar extraction and estimation

Seeds from various stages of desiccation were weighed (in triplicates) and boiled separately for 5 min in 80 % ethanol (3 ml g-1 fresh mass) to inactivate enzymes. The supernatants were separated by centrifugation (1000 g for 20 min) and the leftovers were manually homogenized and extracted three times in boiling ethanol. The residues were rinsed with distilled water after centrifugation and the supernatants were collected. The resulting ethanolic and aqueous supernatants were mixed, evaporated and then redissolved in 10 ml distilled water to form the soluble sugar extracts. Using glucose and sucrose as standards, the Anthrone method (23) and the Phenol sulphuric acid method (24) were used to calculate the quantity of starch, sucrose and total soluble sugar.

Protease assay

Incubation of acetone-purified enzyme extract with substrate (1 % (w/v) casein) was used to determine protease activity. For 3 hrs, the blank and test samples were incubated at 37 °C. After that, 10 % (w/v) TCA was added and the reaction was incubated for 30 min at ambient temperature before being centrifuged for 10 min at 11000 g. The recovered supernatants were then combined with 1 N Folin phenol reagent before being added to 0.2 N (w/v) sodium hydroxide. After 30 min of incubation at 570 nm, the absorbance was measured and represented in units per hr per milligram protein (25).

Determination of antioxidant enzyme activities

Using a chilled pestle and mortar, one gram of fresh seed tissue was ground to a fine powder in liquid nitrogen and then homogenized in 5 ml of ice-cold 100 mM potassium phosphate buffer (pH 7.0), 1 mM ascorbate (in the case of APX), and 2% (w/v) polyvinyl pyrrolidine (PVP). The homogenate was centrifuged at 10000 g for 10 min at 4 °C and the supernatants were collected and employed as an enzyme source in several tests (26) and for protein content measurements. Using Bovine Serum Albumin as a calibration reference, the protein content of the extracts was measured using Lowry's technique.

The activity of catalase (EC 1.11.16) was measured using a reaction mixture that included 1.5 mL phosphate buffer (100 mM, pH 7), 1.2 ml hydrogen peroxide (150 mM) and 300 µl enzyme extract. At 240 nm, there was a drop in absorbance (27). By combining 100 ml enzyme extract, 30 ml H_2O_2 (12.3 mM), 50 ml guaiacol (20 mM) and 3 ml phosphate buffer, the activity of peroxidase (POD, EC 1.11.17) was measured (100 mM, pH 7.0). The mixture's absorbance was measured at 436 nm and POD activity was calculated using a 25 mM^{-1} cm^{-1} extinction coefficient (28). The activity of superoxide dismutase (SOD, EC 1.15.11) was determined using Kono's approach (29). The reaction mixture was made up of 1.3 ml sodium carbonate buffer, 500L NBT and 100 l Triton X-100. The reaction was started by adding 100 µl of hydroxylamine hydrochloride to the mixture. Seventy µl of enzyme extract were added after 2 min. An increase in absorbance at 540 nm corresponded to a % inhibition in the rate of NBT degradation. One unit of enzyme activity is defined as the amount of enzyme concentration required to reduce the absorbance at 540 nm of chromogen synthesis by 50 % in one minute. The enzyme assay contained 0.5 mM ascorbate, 0.4mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7.0), and 50 µl of enzyme extract for a total volume of 1 ml of ascorbate peroxidase (APX, EC 1.11.1.11) activity. The decrease in ascorbate absorbance at 290 nm and 30 °C was used to evaluate APX activity, which was represented as nmol of NADPH oxidized $mg^{-1}$of protein, or mg^{-1} fresh weight (30). The reaction mixture comprised of 1.5 ml of 0.1M sodium phosphate buffer (PH 6.5) and 200 µl of polyphenol oxidase (PPO, EC 1.10.3.2) activity. To begin with the reaction, 0.01 ml catechol was added to the reaction mixture (31). Change in absorbance at 412 nm $minute^{-1}mg^{-1}$protein was used to measure PPO activity. The values obtained with nine measurements carried out on three distinct extracts match to the means SD of the values provided for various assays (three measurements per extract).

Statistical analysis

R Statistical Software (version 4.1.1; R Foundation for Statistical Computing, Vienna, Austria) and SPSS 21.0 software were used for all statistical analyses (v21.0, SPSS Inc.). The study used a completely randomized design with 3 replications for each treatment.

Results

The viability of *G. canarica* seeds is reliant on moisture content, thus after 48 hrs of drying, fresh seeds (Fig. 1) with a moisture content of 28 % and an embryonic axis of 59 % began to deteriorate (Table 1). Within 12 - 15 days of desiccation, seed water content dropped to around 10% and embryonic axis to 22 % and viability was practically lost.

Table 1. Changes in moisture content of seeds and embryo, weight of seeds and embryo and electrolyte leakage in desiccating *G. canarica* seeds

	Days after harvest							
	0	2	3	4	5	8	12	15
Seed MC (%)	28.86±0.17^{h}	27.05±0.38^{g}	23.82±0.16^{f}	21. 66±0.40^{e}	19.37±0.24^{d}	17.55±0.27^{c}	12.94±0.45^{b}	10.72±0.17^{a}
Embryo MC (%)	57.49±1.79^{h}	50.67±1.58^{g}	41.96±2.10^{f}	33.54±1.43^{e}	26.86±1.42^{d}	20.73±1.43^{c}	15.84±0.37^{b}	12.26±1.98^{a}
Seed weight (g)	3.91±0.10^{f}	3.66±0.06^{e}	3.06±0.01^{d}	2.87±0.04^{c}	2.88±0.61^{c}	2.68±0.02^{b}	2.61±0.02^{b}	2.59±0.85^{a}
Embryo weight (g)	0.038 ±0.03^{d}	0.039±0.04^{e}	0.039±0.04^{e,f}	0.040±0.04^{f,g}	0.039±0.02^{g}	0.022±0.02^{c}	0.017±0.01^{b}	0.010±0.01^{a}
Electrolyte leakage(µs/cm/g)	139.90±0.80^{a}	142.70±0.15^{b}	146.80±0.17^{c}	169.60±0.20^{d}	188.23±0.51^{e}	212.03±0.03^{f}	213.06±0.46^{g}	212.66±0.33^{g}

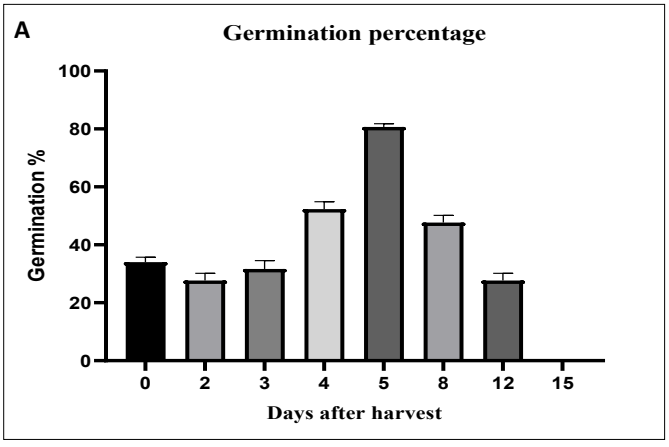
Values are mean of replicates and vertical bars represent ± SD, means within a column followed by same letters are not significantly ($p<0.05$) different as determined by Tukey's test.

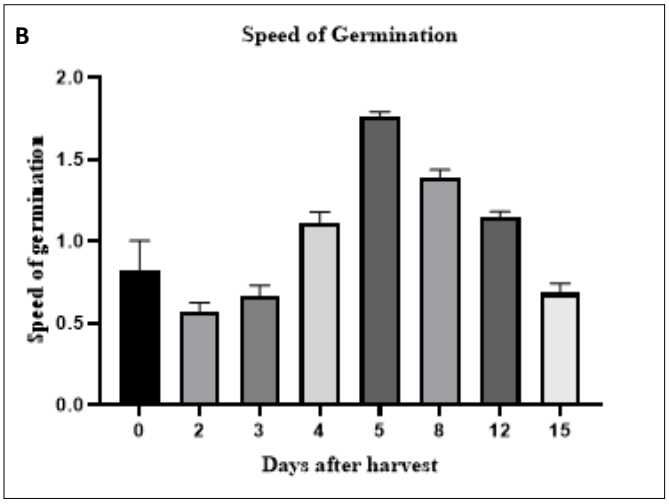
Fig. 1. Freshly collected mature *G.canarica* seeds with red lacinated aril

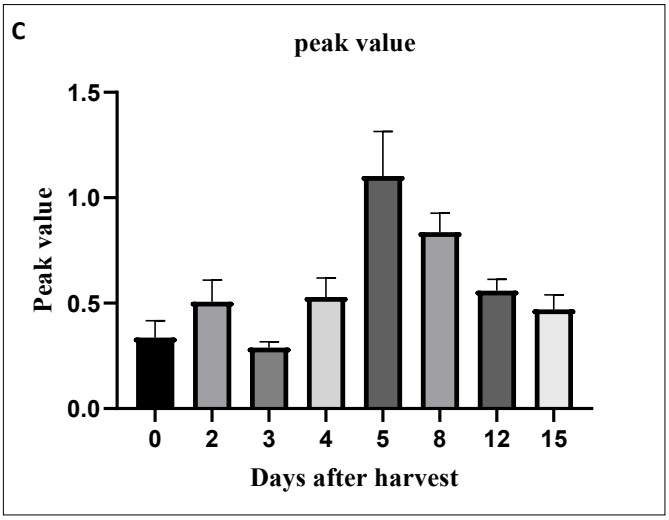
The loss of moisture content caused a progressive decrease in seed weight. In physiologically developed *G. canarica* seeds, the seed coat ratio was 0.21 and the embryo ratio was 0.01. Seed germination in *G. canarica* is hypogeal, and seeds with 28 % Fwt WC showed only 35 % germination, which lasted until the fourth day of harvest (DAH, 22 % Fwt WC).

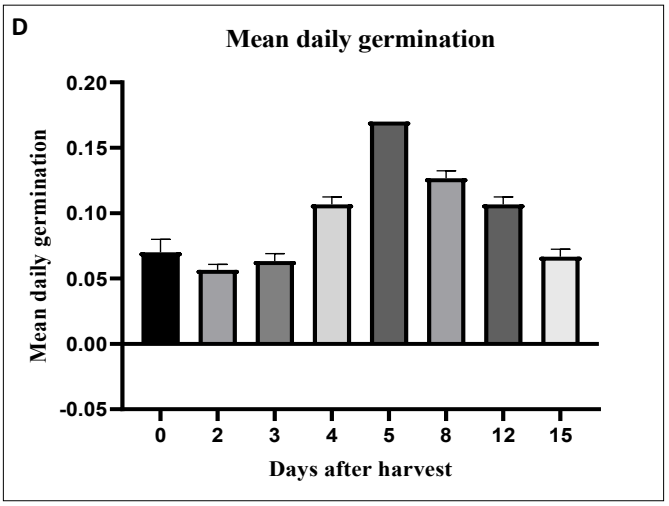
Germination

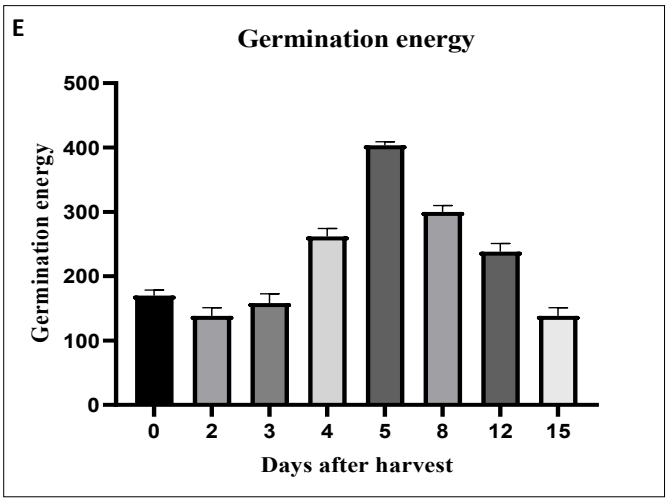
Seed germination in *G. canarica* is hypogeal, and seeds with 28 % Fwt WC showed only 35 % germination, which lasted until the fourth day of harvest (DAH, 22 % Fwt WC). Later, the germination % improved progressively, reaching a peak of 80 %t on the 5th day of harvest with 19.37 % Fwt WC (Fig 2A). Following that, a decrease in germination was observed, and germination was reduced to zero % at 10% Fwt WC (lethal moisture content). As a result, it's possible to confirm that *G. canarica* seeds can withstand desiccation up to a seed moisture content of 19.39 % Fwt WC, which is considered as the critical water content (CWC) for seed viability. The initial germination took an average of 49 days in fresh seeds with a moisture content of 28.86% (control) and seeds dehydrated to critical moisture con











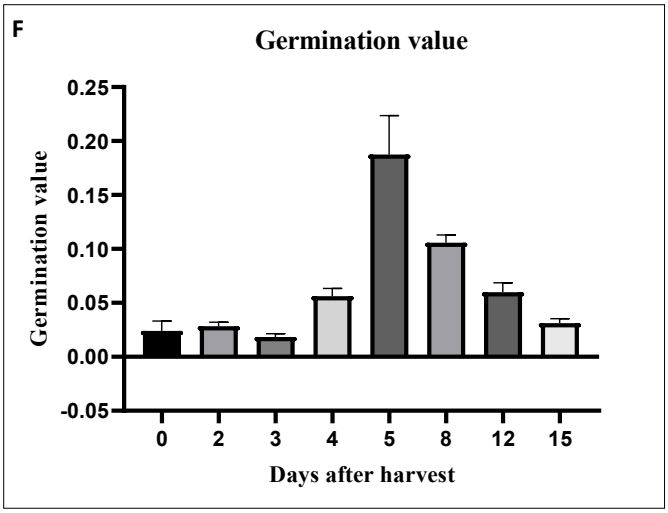


Fig. 2. Germination traits like germination percentage (GP), speed of germination (SPG), peak value (PV), mean daily germination (MDG), germination value (GV) and germination energy (GE) of *G.canarica* seeds during different desiccation periods. Values are mean ± SE and bars are significantly different (P<0.05) among treatments

tent need 40 days of mean germination time (Fig. 3). Seedling growth characteristics such as seedling length, radicle

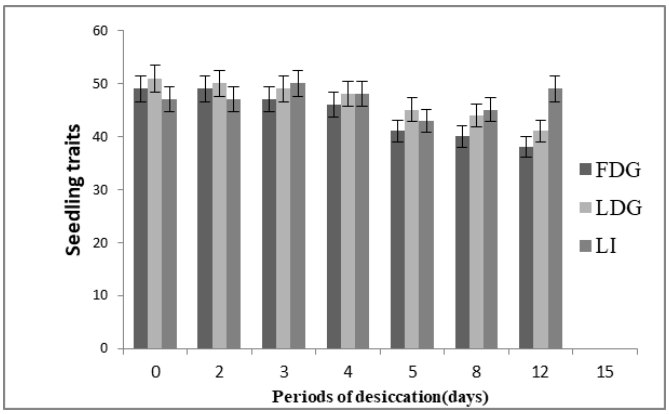


Fig. 3. First day of germination (FDG), last day of germination (LDG) and leaf initiation (LI) in *G.canarica* seedlings in response to desiccation.

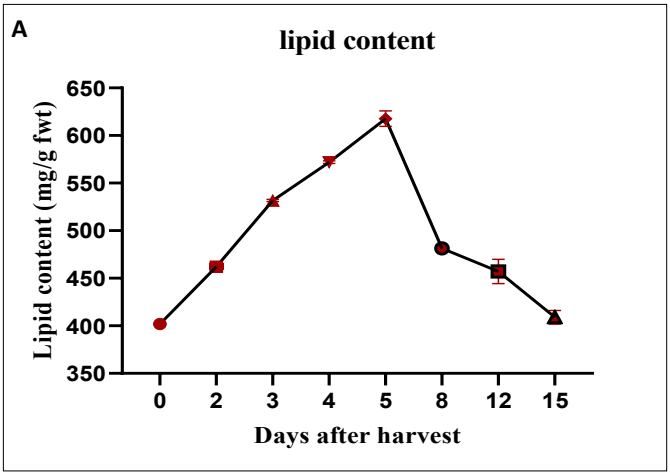
length and plumule length were highest in desiccated seeds with a critical moisture level of 19.37 % moisture content and lowest in freshly harvested seeds with a critical moisture content of 28.86 % moisture content. In 90-day-old seedlings, the maximum seedling length (13.08 cm) and minimum (9.56 cm), plumule length maximum (8.79 cm) and minimum (5.69 cm) and radicle length maximum (3.86 cm) and minimum (4.28 cm) were measured. For seedlings generated from parent seeds dehydrated to a threshold moisture content of 19 %, all growth metrics were at their maximum (Table 2). The strongest significant positive connection between seedling weight and seedling length (r = 0.716**, p=0.000) was seen in seedling weight. The minimum days required for leaf initiation in CMC desiccated seeds are 42-45 days in control seedlings and 45 days in CMC desiccated seeds (Fig. 3). For seedlings with greater germination % desiccation stages, the maximum leaf length (42.50 mm), leaf breadth (17.57 mm) and leaf area (500.05) were achieved. The most significant strong positive connection with seedling length is seen in the seedling vigour index of 90-day-old seedlings (r = 0.926, p=0.000).

Physiological parameters

Electrolyte leakage increased as seeds dried out, and greater dehydration resulted in a considerable increase in leachate conductivity in *G. canarica* seeds (Table 1). The rate of electrolyte loss was inversely proportional to seed moisture content and proportional to MDA levels. During desiccation, the decrease in tetrazolium reduction was accompanied by a decrease in the % of moisture content (Table 3). Freshly collected seeds (0.27), which stain red in the cotyledons and endosperm but light red in the embryo, had higher results. The formazan formation reduced to 0.15 at sixth DAH, when seed MC was 19.39 %, however the embryo showed significant red staining. In non viable seeds, further dehydration to 10.21 % MC on 15 DAH resulted in increased loss of formazan formation and insignificant staining in the embryonic axis and cotyledons. During the early phases of desiccation, memberane peroxidation in the seeds was quite low, with MDA concentration increasing from 1.71 nmol-1g Fwt to 5.10 nmol-1g Fwt (10.21 %).

Biochemical parameters

Desiccation enhanced the total lipid content of *G. canarica* seeds. Freshly harvested seeds have 401.76 mg g^{-1} dwt, and rising lipid content was adversely related with tissue water content until the fifth day of desiccation, lipid content increased rapidly by 617.70 mg g-1 dwt, which coincided with a faster increase in germination %. After that, the lipid content in the seeds was reduced to 380.71 mg g-1 dwt at the end of desiccation (Fig. 4A). Desiccation damage was



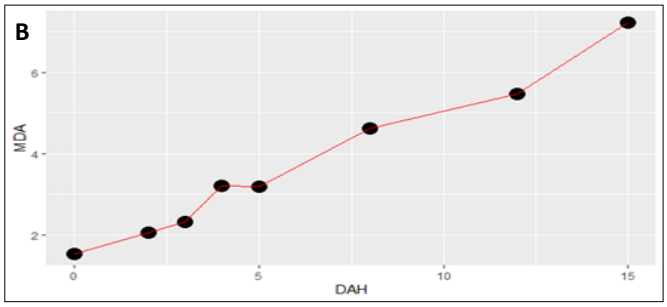


Fig. 4(A). Changes in lipid content during desiccation in *G.canarica* seeds **(B)** Changes of malondialdehyde (MDA) content in *G.canarica* seeds during dessication. Data are mean of six replicates ± SD, significantly at the 0.05 probability level.

considerable once the MC dropped from 19.39 % in seeds. Phenolic and total protein content declined considerably during desiccation, with non-desiccated seeds having the maximum concentrations of 302.54 mg g^{-1} Dwt (Phenol) and 190.70 mg g^{-1} Dwt (Phenol) and 190.70 mg g^{-1} Dwt (Phenol) and 190.70 mg g^{-1} Dwt (Phenol) and 190.70 mg g^{-1} Dwt (Protein). The concentration of total soluble sugars increased dramatically up to the fifth desiccation stage, reaching a maximum of 438.73 mg g^{-1} Dwt. In *G. canarica* seeds, soluble sugars were represented by nearly equal amounts of sucrose throughout the first days of desiccation and sucrose content peaks at the fourth desiccation stage (361.80 mg g^{-1} Dwt) and thereafter declines (RFO). The quantification of starch revealed that seeds were high in this reserve compound at non-desiccating seeds (104.03 mg g^{-1} Dwt), and that further dehydration resulted in a considerable decrease in concentration of 60.32 mg g^{-1} Dwt at 10.21 % MC (Table 4). The total free amino acid content of seeds increased as a result of desiccation stress; initially,

Table 2. Seedling length, Root length, Shoot length, seedling vigour index, seedling weight, petiole length, leaf length, leaf width and leaf area of 90 days old *G. canarica* seedlings in response to seed desiccation

Days after harvest	Seedling length (cm)	Root length (cm)	Shoot length (cm)	Seedling vigour index(SVI)	Seedling weight(g)	Petiole length (mm)	Leaf length (mm)	Leaf width (mm)	Leaf area(mm^2)
0	9.56±0.11^{a}	3.86±0.01^{b,c}	5.69±0.110^{a}	287.05 ± 18.53^{a,b}	9.56±0.11^{a}	3.84±0.82^{c}	28.35 ± 0.32^{a}	13.18 ± 0.32^{b}	249.56 ± 8.62^{b}
2	10.24±0.02^{c}	3.66±0.03^{a}	6.57±0.045^{c}	317.44 ±13.53^{b,c}	10.24±0.02^{c}	3.70±0.37^{b,c}	28.90 ± 0.30^{a}	10.88 ± 0.11^{a}	209.35 ± 3.61^{a}
3	10.13±0.02^{b,c}	3.90±0.03^{c}	6.23±0.035^{b}	329.44 ±11.66^{c}	10.13±0.02^{b,c}	2.92±0.21^{a,b}	33.65 ± 0.18^{b}	13.01 ± 0.14^{b}	292.20 ± 4.39^{c}
4	12.42±0.03^{e}	3.93±0.12^{c}	8.48±0.141^{d}	650.18 ± 12.81^{d}	12.42±0.03^{e}	4.68±0.33^{d}	29.83 ± 0.49^{a}	17.37 ± 0.14^{d}	345.73 ± 7.54^{d}
5	13.08±0.019^{f}	4.28±0.05^{d}	8.79±0.054^{e}	1055.23 ±4.78^{d}	13.08±0.019^{f}	5.23±0.13^{d}	42.50 ± 0.23^{d}	17.57 ±0.20^{d}	500.05 ±5.15^{f}
8	12.16±0.025^{d}	3.83±0.04^{a,b,c}	8.32±0.028^{d}	579.60±10.17^{d}	12.16±0.025^{d}	3.24±1.4^{a,b,c}	40.38 ± 1.15^{c}	15.68 ± 0.57^{c}	413.81 ±22.79^{e}
12	9.95±0.10^{b}	3.71±0.02^{a,b}	6.24±0.095^{b}	275.64±10.67^{a}	9.95±0.10^{b}	2.74±0.37^{a}	28.90 ± 0.09^{a}	14.96 ± 0.06^{c}	258.10 ±21.21^{b,c}
F value	493.35***	11.53***	239.30***	541.53**	493.35***	10.39***	135.30***	73.45***	63.83***

***Significant at p<0.001 level; Means within a column followed by same letters are not significantly (p<0.05) different as determined by Tukey's test.

Table 3. Viability loss of *G. canarica* seeds during different desiccation periods

Days after	Moisture content	Formazan for-	Visual observation
0	28.86 ± 0.17	0.27 ± 0.004	Cotyledons and endosperm stains red, embryo light red.
2	27.05 ± 0.38	0.27 ± 0.004	Cotyledons, endosperm, embryo stains red.
3	23.82 ± 0.16	0.26 ± 0.003	Cotyledons ad endosperm pale red, embryo red.
4	21. 66 ± 0.40	0.19 ± 0.007	Cotyledons and endosperm light red with unstainable margins, embryo red.
5	19.37 ± 0.24	0.15 ± 0.006	Endoperm and cotyledons not stained, embryo deep red.
8	17.55 ± 0.27	0.13 ± 0.001	Endoperm and cotyledons mot stained, very feebly stained embryo with unstainable margins.
12	12.94 ± 0.45	0.09 ± 0.003	Endoperm and cotyledons not stained, embryo shows very pale red in small patches
15	10.72 ± 0.17	0.27 ± 0.004	Cotyledons and endosperm stains red, embryo light red.

Table 4. Biochemical changes during desiccation of *G. canarica* seeds

Period of desiccation	Moisture content	Total soluble sugar (mg/g dwt)	Starch (mg/g dwt)	Sucrose (mg/g dwt)	Phenol (mg/g dwt)	Amino acid (mg/g dwt)	Proline (mg/g dwt)	Protease(units $h^{-1}mg^{-1}$protein)
0	28.86 ± 0.17^{h}	258.23±2.51^{a}	104.03±1.85^{g}	235.42±0.83^{d}	302.54±1.58^{h}	26.44±0.67^{a}	3.84±0.05^{f}	0.13 ±0.01^{a}
2	27.05 ± 0.38^{g}	292.72±3.49^{b}	96.13±0.69^{f}	261.43±1.49^{e}	284.35±4.28^{g}	32.59±1.10^{b}	3.55±0.05^{e}	0.17 ± 0.007^{b}
3	23.82 ± 0.16^{f}	380.26±3.33^{f}	89.6±2.31^{e}	325.36±1.75^{g}	250.96±2.31^{f}	36.55±0.82^{c}	4.67±0.03^{g}	0.22 ±0.008^{c}
4	21. 66 ± 0.40^{e}	425.06±3.91^{g}	79.19±1.19^{d}	361.80±1.74^{h}	224.50±2.51^{e}	58.82±0.89^{d}	3.53±0.04^{e}	0.25 ±0.01^{d}
5	19.37 ± 0.24^{d}	438.73±2.70^{h}	71.12±0.71^{c}	296.36±1.30^{f}	201.64±2.04^{d}	61.93±0.95^{e}	3.39±0.03^{d}	0.28 ±0.04^{e}
8	17.55 ± 0.27^{c}	370.02±2.62^{e}	63.95±12.4^{a,b}	229.77±1.87^{c}	196.00±1.63^{c}	76.29±0.73^{g}	2.34±1.00^{c}	0.32±0.01^{f}
12	12.94 ± 0.45^{b}	363.71±2.74^{d}	66.51±1.31^{b,c}	193.84±1.17^{b}	172.72±4.24^{b}	77.82±0.44^{h}	2.23±0.05^{b}	0.36±0.08^{g}
15	10.72 ± 0.17^{a}	302.25±2.76^{c}	60.32±1.28^{a}	132.70±1.38^{a}	158.67±3.64^{a}	70.08±0.71^{f}	1.24±0.04^{a}	0.39±0.01^{h}
F value	442.03***	2661.89***	73.88***	14722.57***	1814.00***	2792.28***	3679.57***	156.09***

***Significant at p<0.001 level; Means within a column followed by same letters are not significantly (p<0.05) different as determined by Duncan's test.

the seed had 26.44 mg g^{-1} Dwt, and the greatest amino acids were accumulated under desiccation stress (77.8 mg g^{-1} Dwt) at 13.05 % MC. Freshly harvested seeds have a proline content of 3.84 mg g^{-1} Dwt, which increases until the third day of desiccation, after which there is a considerable drop in proline concentration towards the conclusion of desiccation (Table 4). Parallel to seed desiccation, a noticeable increase in protease activity was found (Table 4). In comparison to fresh seeds of 28.82 % MC, the maximum activity of protease was seen in non viable seeds of 15 DAH (10.72 % MC). Protease had a positive correlation with seed desiccation periods ($r = 0.943$, $p<0.05$), but was inversely associated to seed MC ($r = -0.970$, $p<0.05$).

Antioxidant profile during seed desiccation

SOD activity was 0.230 Unit, mols mg-1 protein in fresh seeds without desiccation, and increased to 0.59 Unit, mols mg^{-1} protein with continuous desiccation (Fig. 5a). Catalase enzyme activity remained modest during seed desiccation, rising ahead of SOD from 0.016 mol H_2O_2 min-1mg-1 protein to 0.318 mol H_2O_2 $min^{-1}mg^{-1}$ protein on the third day of desiccation and then declining sharply (Fig. 5b). The activity of APX, an enzyme involved in the ascorbate–glutathione cycle, reduced progressively during the initial phases of desiccation before increasing after seed MC fell to 19.37% (Fig. 5c). PPO enzyme showed a similar trend, with maximum enzyme activity observed under extreme desiccation stress (Fig. 5d). The peroxidase enzyme was the most active during desiccation in seeds, with activity decreasing two days later. After the seeds attain CMC (19.37 %), the maximal POX activity (249.96 mol $min^{-1}mg^{-1}$ protein) was detected (Fig. 5e).

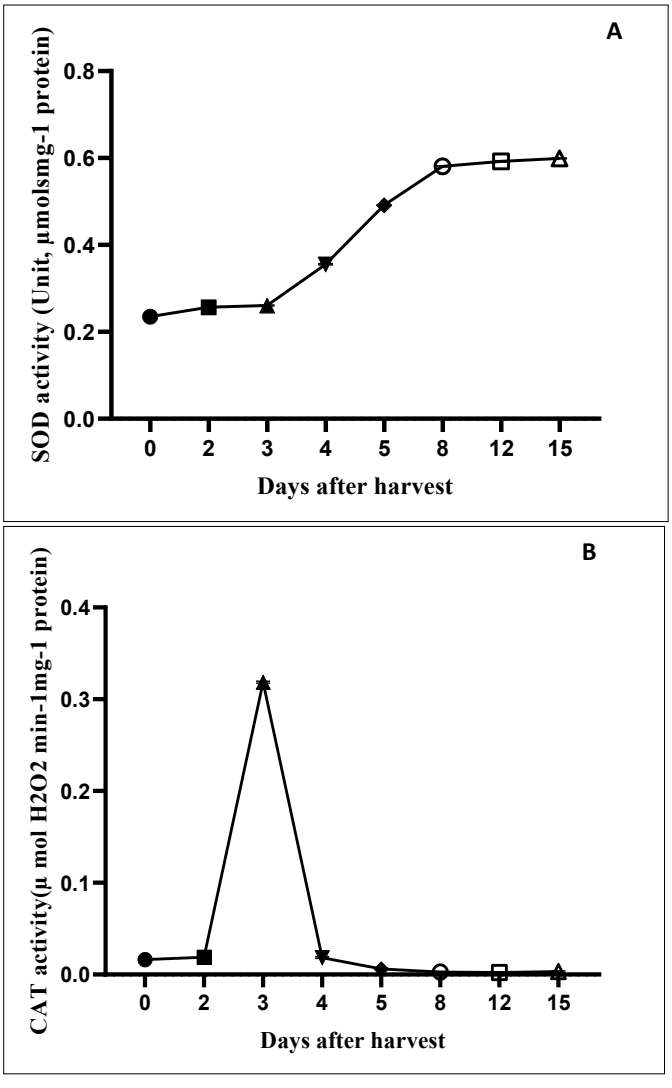


Fig. 5. Graph showing the activities of a. Superoxide dismutase b. Catalase c. Ascorbate peroxidase d. Polyphenol oxidase e. peroxidase in seeds of *G.canarica*. Lines represent the mean values ± standard error obtained for three replicates

Discussion

Recalcitrant seeds are resistant to dryness and sub-zero temperatures and they can't be saved using traditional gene bank methods (32). When the moisture content of the seeds fell below 28 % during desiccation, the seeds quickly lost viability, demonstrating their actual refractory nature and the seed became completely non viable when dehydrated below the crucial moisture content of 10 % FM WC on 15 DAH. In Myristicaceae species, there was a high degree of critical moisture content and numerous desiccation sensitive tree species seeds had a reasonably high CMC (33-35). When the embryo is dehydrated to about 26.86 %, it loses viability. *G. canarica* seeds have a CWC of 19.37 %, which is consistent with that of an earlier study revealing that seeds of *Myristica malabarica* with 27 % MC lost viability after a week in open dry conditions (36). Natural seeds have a low germinability, indicating that germination was problematic for this tree species. Harvesting and storing seedlings at the appropriate times is therefore critical for producing healthy seedlings. *G. canarica* had a tiny embryo that was not linked to the seed coat, which was covered by a brown ruminated layer typical of Myristicaceae species (37). At the moment of shedding, seeds had a very poor seed coat and embryo ratio (0.2), according to the current study. For 104 woody plants, an average value of 0.209 (38) was determined. The seed coat contribution to whole seed weight was found to be minimal in 34 refractory seeds from Eastern Australian rainforests and seeds disseminated with high moisture content invest significantly less in seed coats, reflecting the genuine recalcitrant nature of seeds (39, 40). Due to their porous comparatively thin seed coat, Seeds are capable of rapid water loss during natural drying and comparable observations were obtained in Horse chestnut seeds (41). Seed germination and seedling establishment are critical phases in the plant growth cycle, since they influence and determine species survival in natural habitats. Germination started in between 45-47 days and was characterized by radicle protrusion through the testa and lower germination in freshly collected seeds may be due to an under developed embryo. Recalcitrant seeds of *Zigadenus desnsus* and *Z. lemanthoides* are also recorded under developed embryo and are classified as showing morphological dormancy (42). Germination is found to be highly erratic in *G. canarica* seeds and in our study, seeds that subjected to desiccation up to 5 days in open laboratory conditions shows maximum germination % than the freshly collected seeds and have significant positive correlation with speed of germination, peak value, mean daily germination, germination value and germination energy, as well as the lowest values for average time for radicle and shoots emission. The vigour test applied to *G. canarica* seeds (seedling vigour index, plumule length, radicle length, seedling weight) also showed highly significant correlation with total germination. Moisture content of fresh seeds and rate of reduction in moisture content as well as germination are not comparable with the characteristics of recalcitrant seeds. Such erratic germination behavior were reported in *Corypha umbraculifera* (43). The loss of viability during desiccation has been linked to a loss of cell membrane integrity and increased cell membrane permeability (44) was observed in *G. canarica* seeds throughout the desiccation process, with a strong association between seed moisture content and MDA accumulation. MDA is the end result of lipid memberanes peroxidation in plants, and it can directly reflect cell damage (45). Reports are on the viability loss in refractory seeds in connection with lipid peroxidation in various species (46). Desiccation-induced damage leads to an excess of reactive oxygen species (ROS) and lipid peroxidation (47, 48). A similar condition was found in *G. canarica* seeds, where ROS appears to have caused damage to memberane lipids, resulting in viability loss.

Our findings are consistent with the observation of dehydrated *Knema attenuata* seeds, another myristicacean species (49). Tetrazolium activity in dehydrated recalcitrant seeds with the electron transport chain coincided in general with the results of the germination assay and tetrazolium assays, which demonstrated a precipitous drop in viability as drying progressed (50). The degradative processes were sufficiently high in the present study on desiccated seeds of *G. canarica* for electron transport to decline in tandem with other deleterious processes, leading to viability loss at a lower water content, similar to what was observed in recalcitrant seeds of *Zizania palustris* (51). Desiccation was found to impact the physiological and biochemical changes in resistant seeds in *Shorea robusta* (52) and *Telfairia occidentalis* (53). Lipids are non-polar hydrocarbons that are water insoluble when contrasted to polar molecules such as glucose and sucrose. *G. canarica* seeds have a high lipid content and those initially stored lipids stay non-polar, allowing them to float in marshy conditions; additionally, it prevents water from entering the germplasm through the testa (54). Non-polar lipids, on the other hand, hinder water from entering protoplasm and consequently seed germination in freshly collected non-desiccated seeds.

Until *G. canarica* seeds dehydrate, stored lipids are absorbed and lipid content declines as the seeds reach critical moisture content with maximum germination %. Sucrose is the most abundant soluble carbohydrate found in ripe seeds of many species and it serves as a substrate for metabolic activities that occur at low temperatures (55). It is assumed to play a key role in cellular desiccation tolerance (56). However, sugar was discovered in considerable amounts in *G. canarica* seeds regardless of desiccation tolerance and the sucrose content was not connected to seed desiccation sensitivity.

This finding backs up that soluble carbohydrates alone do not promote seed desiccation tolerance (57). In the current investigation, it was fascinating to dis-

cover that desiccation of *G. canarica* seeds resulted in an increase in soluble sugars. It could have been generated by starch breakdown into simple sugars, as evidenced by biochemical changes and similar findings have previously been described in *Theobroma cacao* and *Camellia sinensis* (58). In ripe seeds, phenolic chemicals tend to react with mitochondria, inhibiting ATP generation (59). High phenolic content during seed dissemination appears to diminish with desiccation, and increased phenolic concentration after seed dispersal shows that such substances may be responsible for prolonging germination in seed banks (60). Desiccation studies on *G. canarica* seeds revealed a strong link between seed viability and total phenols, suggesting that total phenols may play an important role in the prevention of ageing. As reported in seeds, the amount of protein is likewise closely linked to longevity (61, 62). Protein content in desiccating seeds fell significantly (P0.05) with a positive association (r = 0.938, P0.05) in our study. Seeds with low protein content have a lower vigour (63) and are less able to tolerate desiccation (64). During dehydration, *G. canarica* seeds showed a significant decrease in soluble proteins and an increase in total free amino acids, implying that amino acids are produced by the mobilization of storage proteins in mature seeds during desiccation and may be linked to the role of these compounds as osmolytes, which are needed to complete the germination process. For biosynthesis and energy generation, proteins that have been produced and stored in mature seeds are broken down into free amino acids (65).

Proline is an essential amino acid involved in drought stress prevention and is considered an osmo protectant (66-68). Proline participates in ROS protection during desiccation by decreasing lipid peroxidation and H_2O_2 concentration while enhancing antioxidant enzyme activity (69). Proline activity decreased in tandem with water content in the current investigation and comparable results were observed during desiccation of refractory *Camellia sinensis* seeds (70). *G. canarica* seeds are abrasive and dehydration-induced viability loss could be connected to a reduction in proline synthesis. Because there is no free water available during seed desiccation, non-enzymatic processes such as lipid peroxidation are anticipated to play a role in ROS accumulation (71, 72). Membranes are connected with low molecular weight antioxidants, and their major purpose is to prevent peroxidation (73). ROS, particularly H_2O_2, is an important signal in a variety of physiological activities (74, 75). In the current study, there was an increase in both SOD activity and H_2O_2 level in cells during desiccation, which boosted the activity of antioxidant enzymes to eliminate ROS than dehydrated seeds, which was followed by an increase in desiccation stress. Peroxidase and ascorbate peroxidase would increase with increased SOD activity, however in *G. canarica*, those enzymes' activity reduced in the early stages compared to dried seeds, followed by an increase in desiccation stress. However, an increase in catalase and polyphenol oxidase activity during desiccation may be responsible for the elimination of excess ROS caused by stress during seed damage (76). The cumulative ROS damage caused by desiccation was a major cause of cell membrane structural integrity loss and seed viability loss in the seeds. Desiccation increased viability loss due to loss of membrane integrity in the seeds, which was linked to ROS formation and associated lipid peroxidation products in this study. In conclusion, multiple antioxidant systems are discovered to be simultaneously and negatively operational with the viability of desiccating recalcitrant *G. canarica* seeds during desiccation.

Conclusion

G. canarica (King) Warb. is an endemic tropical tree species, highly habitat specific with very poor natural seed germination. Our findings shows the seed storage behavior of *G. canarica* are recalcitrant and quickly lose their viability. Biochemical study shows high lipid content on mature seeds at the time of shedding could be an adaptation strategy to float in water filled forest floor. But the dehydration increased germination percentage in *G. canarica* seeds to a maximum of 80% and needs further study to understand the mechanisms causing germination rise.

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Authors contributions

Anusha collected, performed the experiments, assemblage of data and wrote the manuscript. Anilkumar and Gangaprasad gave critical revision of the article for important intellectual content, edited the manuscript and final approval of the article.

Compliance with ethical standards

Conflict of interest: All authors declare that they have no conflicts of interest.

Ethical issues: None.

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