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EFFECT OF DORMANCY BREAKING TREATMENTS ON GERMINATION , VIGOUR AND PHYSIO-BIOCHEMICAL CHANGES IN SEEDS OF *RAUVOLFIA SERPENTINA* (L.) Benth. ex Kurz–ENDANGERED MEDICINAL SPECIES

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Rauvolfia serpentina (L.) Benth. ex Kurz is an endangered medicinal species which produces large proportion of non-viable seeds. In addition, the viable seeds show dormancy because of adverse influence of the stony endocarp, seed coat induced dormancy and non-deep physiological dormancy rendering the seed mediated propagation unsatisfactory . The present study indicates the effect of dormancy breaking treatments on germination and underlying physio-biochemical changes in *Rauvolfia serpentina* (L.) Benth. ex Kurz. Untreated seeds did not show germination, whereas, the maximum germination percentage (36.67%) was observed in seeds subjected to GA_3 200 ppm for 48 hrs, which was closely followed by GA_3 100 ppm for 48 hrs (28.89%), KNO_3 1% for 48 hrs (23.33%) and chilling at 5^0C for 4 weeks as well as thiourea 0.5% for 48 hrs (22.22%). Henceforth, above seed treatments can pave the way for breaking dormancy ; inducing germination and improving vigour of seeds of *Rauvolfia serpentina* which otherwise remained ungerminated. Physio-biochemical responses of seeds to these pre-sowing treatments have also been discussed.

Keywords: Germination percentage, Sarpagandha, Mean germination time, physio-biochemical responses , Seedling vigour index

Rauvolfia serpentina commonly known as Sarpagandha belonging to family Apocynaceae is an important critically endangered medicinal plant species which grows generally upto an altitude of 1000 m amsl. It has been reported to contain 50 indole alkaloids that are mainly localized in the root bark and used for the treatment of various central nervous system disorders associated with psychosis, schizophrenia, insanity, insomnia and epilepsy. It is an important medicinal plant of Indian subcontinent and South East Asian countries. Poor seed viability, low seed germination percentage, low vegetative propagation rate, indiscriminate collection and overexploitation from natural habitat for commercial purposes has rendered *Rauvolfia serpentina* to be threatened with extinction even though the seeds are chosen for sowing purpose (Dey and De 2011, Mittal *et al.* 2012). The seed mediated propagation is unsatisfactory due to production of large proportion of non-viable seeds (Mitra 1976) and dormancy because of adverse influence of the stony endocarp, seed coat induced dormancy and non-deep physiological dormancy all of which result in very low (5 - 30%) germination percentage (Hussain and Jha 2014). Another serious limitation for seed germination of this species is the absence of embryos, though the fruits may appear perfectly normal externally. Propagation by direct sowing of seeds in the field has not been found successful. Several chemical and or physical treatments have been found very effective in breaking seed dormancy due to hard seed coat and other physiological factors , thereby improving the germination ability of seeds (Thakur 2008, Thakur and Himangini 2015, Bhardwaj *et al*. 2016 , Negi *et al*. 2017). Further, key Physio-biochemical phenomena like mobilization of stored reserves, imbibition , phenol content and other biochemical changes play a crucial role during seed germination (Yang *et al* 2016 ,Tejavathi *et al*. 2017, Zhao *et al*. 2018). Present studies were, therefore undertaken with the objective to induce germination and vigour of seeds by dormancy breaking treatments and also to knowhow the effects of these treatments on underlying physio-biochemical changes in seeds of *Rauvolfia serpentina*.

MATERIALS AND METHODS

Seeds of *Rauvolfia serpentina* were procured from Herbal garden Neri (1000 m amsl) during the month of May. Thereafter, seeds were cleaned and air dried at room temperature and

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tested for seed viability (Tz) and moisture content (%) which showed 50% seed viability and 8.23% moisture content. Further, in order to study the effect of presowing treatments on germination and vigour, seeds were surface sterilized with 0.1% mercuric chloride for 2 minutes to remove bacterial and fungal strains, thoroughly washed with distilled water thrice to remove the strains of mercuric chloride completely.These seeds were subjected to eleven pre-sowing seed treatments viz., chilling at 5ºC for 4 weeks, scarification with H_2SO_4 for 15 mins, mechanical scarification followed by 100 ppm gibberellic acid (GA_3) for 12 hrs, mechanical scarification followed by GA_3 200 ppm for 12 hrs, KNO_3 1% for 24 hrs, KNO_3 1% for 48 hrs, GA_3 100 ppm for 48 hrs, GA_3 200 ppm for 48 hrs, thiourea 0.5 % for 24 hrs and 48 hrs along with one set of untreated seeds to serve as control respectively. Thereafter, treated seeds in four replicates of 30 seeds each were allowed to germinate in petri dishes lined with Whatman No.1 filter paper, using top paper method in seed germinator at 35 ± 2^0C and 80 % RH under 16 hrs light and 8 hrs dark periods. Daily count of germination was taken and final count was recorded on 90thday. The experiment was conducted in CRD. Germination percentage and other attributes were tested according to ISTA (1965). The formulae used are as follows:

$$\text{Mean germination time (MGT days)} = \sum \frac{(ni \times d}{N}$$

Where, ni is the number of germinated seeds on day i; d is the incubation time (day); N is the total number of seeds germinated.

Germination speed: Daily count of normally germinated seeds was made upto 90thday from the day of sowing. The speed of germination was calculated by using the following formula, suggested by Maguire (1962).

$$\text{Speed of germination} = \frac{n1}{1} + \frac{n2}{2} + \cdots \frac{nx}{x}$$

Where, n_1, n_2, n_x are the number of seeds germinated on day 1st to 90th day and 1, 2, 3---x are the number of days

$$\text{Germination energy} = \frac{\text{Germination percentage}}{\text{Days of completion of germination}}$$

Seedling vigour indices-I and II were calculated as per the following formula given by Abdul Baki and Anderson (1973).

Seeding vigour index- I = Germination (%) x Seedling lenght (cm)

Seeding vigour index- II = Germination (%) x Seedling dry weight (g)

Imbibition capacity (%): Pre-weighed seeds were immersed in the beaker containing distilled water. The seeds were allowed to imbibe water at room temperature i.e. 32°C ± 2. Thereafter, these seeds were removed, wiped with absorbent paper and weighed, upto 9 hrs after every 3 hr interval. This method is modified method described by Kandari *et al*. (2008).

Mobilization efficiency (%): Seed samples after recording their initial dry weight were allowed to germinate in Petri dishes in seed germinator at optimum temperature (25 ^{0}C ± 2^{0}C) till the completion of germination. Thereafter, seed remnant was dried separately at room temperature for 24 hrs (Shrivastava and Sareen, 1974). Mobilization efficiency was calculated by following formula:

$$\text{Mobilization efficiency} = \frac{\text{Dry wt of original seed - Dry wt of seed remnant}}{\text{Dry wt of the original seed}} \times 100$$

Total soluble sugars were determined as per the method of Dey (1990) using alcohol as extraction medium and total phenol content were estimated according to Singleton *et al.* (1999) using ethyl alcohol as extraction medium.

Alpha-amylase activity was assayed by extracting the enzyme in Tris maleate - NaOH buffer and estimated according to Filner and Varner (1967). Protease activity was determined according to the method given by McDonald and Chen (1965). Total dehydrogenase activity was determined as per Bialecka and Kepczynski (2008). The data was analyzed by Completely Randomized Design

(CRD).

RESULTS AND DISCUSSION

Germination and vigour characteristics:

Untreated (control) seeds did not show germination, whereas All the eleven treatments were found effective in breaking seed dormancy and inducing seed germination and vigour (Table 1& 2). Table 1 reveals that seeds treated with GA_3 200 ppm for 48 hrs registered maximum germination percentage (36.67%) coupled with earliest onset of germination (9.33 days). Other beneficial dormancy breaking treatments which resulted in higher germination percentage were GA_3 100 ppm for 48 hrs (28.89%), KNO_3 1% for 48 hrs (23.33%) and chilling at 5^0C for 4 weeks as well as thiourea 0.5% for 48 hrs (22.22%). Shortest mean germination time (25.63 days) was observed in seeds pretreated with GA_3 100 ppm for 48 hrs (Table 1). Acid scarification for 15 min resulted in 21.11 % germination, however, the onset of germination was relatively late (70 days). Similar trend was recorded for germination speed and germination energy as all above treatments resulted in higher speed and energy of germination.

Table 2 reveals that the maximum seedling dry weight (0.010 g) was obtained for seeds pretreated with GA_3 100 ppm for 48 hrs ,whereas the maximum seedling vigour indices –I (230.12) and II (0.330) were obtained for seeds invigorated with GA_3 200 ppm for 48 hrs . This was followed by GA_3 100 ppm for 48 hrs , KNO_3 1% for 48 hrs (23.33%) and chilling at 5^0C for 4 weeks as well as thiourea 0.5% for 48 hrs . Similar trend was observed for seedling dry weight, seedling vigor index I and Seedling vigor index II indicating that above treatments were helpful not only in breaking dormancy and inducing germination but these were able to produce vigorous seedlings in terms of size and dry matter.

PHYSIO-BIOCHEMICAL CHARACTERISTICS

Table 3 reveals that the maximum imbibition capacity (3.31%) and mobilization efficiency (63.87%) were obtained in seeds pretreated with GA_3 200 ppm for 48 hrs which were closely followed by seeds pretreated with GA_3 100 ppm for 48 hrs, KNO_3 1% for 48 hrs and thiourea 0.5% for 48 hrs i.e. 54.44, 49.33 and 45.67% respectively. One percent KNO_3 for 48 hrs was statistically at par with thiourea 0.5% for 48 hrs with regard to mobilization efficiency. Interestingly, untreated seeds showed least (1.05%) imbibition capacity and zero mobilization efficiency. Pattern of changes in the activity of hydrolases, total soluble sugars and phenol content as affected

Table 1: Effect of seed invigoration treatments on germination characteristics of seeds of *Rauvolfia serpentina*

Treatments	Onset of germination (days)	Germination percentage (%)	Mean germination time (days)	Speed of germination	Germination energy
Control	0.00	0.00	0.00	0.000	0.000
Chilling (5^0C) for 4 weeks	27.33	22.22 (28.01)	37.51	0.110	0.503
Scarification with H_2SO_4 for 15 mins	70.00	21.11 (27.32)	73.14	0.116	0.273
Mechanical scarification followed by GA_3 100 ppm for 12 hrs	16.00	18.89 (25.68)	29.34	0.202	0.439
Mechanical scarification followed by GA_3 200 ppm for 12 hrs	29.33	20.00 (26.50)	45.26	0.117	0.322
KNO_3 1% for 24 hrs	34.67	21.11 (27.32)	48.99	0.133	0.334
KNO_3 1% for 48 hrs	32.00	23.33 (28.83)	43.43	0.169	0.392
GA_3 100 ppm for 48 hrs	11.33	28.89 (32.49)	25.63	0.355	0.700
GA_3 200 ppm for 48 hrs	9.33	36.67 (37.23)	28.14	0.427	0.769
Thiourea 0.5% for 24 hrs	48.33	20.00 (26.50)	66.58	0.084	0.242
Thiourea 0.5% for 48 hrs	31.67	22.22 (28.09)	46.20	0.043	0.396
C. $D_{0.05}$	2.84	2.76	4.89	0.039	0.120

Table 2: Effect of seed invigoration treatments on seedling vigour in *Rauvolfia serpentine*

Treatments	Seedling dry weight (g)	Seedling vigor index I (length)	Seedling vigor index II (mass)
Control	0.000	0.00	0.00
Chilling (5^0C) for 4 weeks	0.006	65.59	0.133
Scarification with H_2SO_4 for 15 mins	0.004	43.40	0.084
Mechanical scarification followed by GA_3 100 ppm for 12 hrs	0.008	40.10	0.151
Mechanical scarification followed by GA_3 200 ppm for 12 hrs	0.006	34.67	0.120
KNO_3 1% for 24 hrs	0.007	48.19	0.148
KNO_3 1% for 48 hrs	0.008	69.43	0.189
GA_3 100 ppm for 48 hrs	0.010	146.48	0.289
GA_3 200 ppm for 48 hrs	0.009	230.12	0.330
Thiourea 0.5% for 24 hrs	0.006	45.00	0.120
Thiourea 0.5% for 48 hrs	0.007	56.07	0.156
C. D0.05	N/S	31.65	0.011

by various treatments is enumerated in table 4 indicating that maximum alpha-amylase, protease and dehydrogenase activities were induced by GA_3 200 ppm for 48 hrs which were 3-4 fold higher as compared to the control. Other effective treatments which resulted in 2-3 fold rise in activities of above enzymes over the control were GA_3 100 and 200 ppm for 48 hrs, KNO_3 1% for 24 and 48 hrs and thiourea 0.5% for both durations. Untreated (control) seeds registered lowest activities of these enzymes coupled with least total soluble sugars and highest phenol content . Whereas maximum sugars (30 fold) and lowest phenol contents were registered by seeds treated with GA_3 200 ppm for 48 hrs. Similar trend of higher total soluble sugars and lower phenol content was also shown by other beneficial treatments.

The data suggest the beneficial effects of presowing seed treatments in overcoming dormancy which may be attributed to changes in seed coat ultra structure (Solichatun et.al.2016), membrane repair, increased protein synthesis and more efficient mobilization of sugars and proteins (Srinivasan *et al.* 1999). Out of ten treatments, gibberellins were found most effective in breaking seed dormancy, advancing and enhancing seed germination and vigour. The role of gibberellins in breaking

Table 3: Effect of seed invigoration treatments on mobilization efficiency and imbibition capacity in seeds of *Rauvolfia serpentina*

Treatments	Imbibition capacity (%)	Mobilization efficiency (%)
Control	1.05	0.00
Chilling (5^0C) for 4 weeks	1.34	28.21
Scarification with H_2SO_4 for 15 mins	1.65	25.33
Mechanical scarification followed by GA_3 100 ppm for 12 hrs	1.46	24.67
Mechanical scarification followed by GA_3 200 ppm for 12 hrs	1.76	20.00
KNO_3 1% for 24 hrs	1.95	45.10
KNO_3 1% for 48 hrs	2.48	49.33
GA_3 100 ppm for 48 hrs	2.75	54.44
GA_3 200 ppm for 48 hrs	3.31	63.87
Thiourea 0.5% for 24 hrs	1.98	26.82
Thiourea 0.5% for 48 hrs	2.35	45.67
C. $D_{0.05}$	0.47	3.66

seed dormancy has been studied extensively and it is well established (Bewley and Black, 1994). Gibberellins are known to obviate the requirement of seeds for various environmental cues, promote germination by triggering enzyme activity and counteracting the inhibitory effects of ABA. Gibberellic acid and other seed invigoration treatments improve and repair embryo disorders and other internal conditions of the seeds including many physio-biochemical changes and thereby induce the germination of seeds alongwith enhanced seedling vigour. In addition underlying key Physiological processes like mobilization of stored reserves, imbibitions capacity, lowered phenol content and other biochemical changes play a crucial role during seed germination (Yang *et al.* 2016, Tejavathi *et al*. 2017, Zhao *et al* 2018;). Improved germinability of seeds by presowing seed treatments is also associated with higher antioxidant enzyme activities (Bailly *et al*. 2000). Our earlier work has also shown that GA_3 induced the germination and vigour of even the aged non-germinable seeds of *Withania somnifera* (Thakur and Himangini 2015). Present studies revealed substantial improvement in germination characteristics

Table 4: Effect of seed invigoration treatments on enzyme activities, total soluble sugars and total phenol contents in seeds of *Rauvolfia serpentine*

Treatments	Alpha-amylase (mg starch degraded min^{-1} g^{-1} fresh weight)	Protease (µg amino acid released $hour^{-1}$ g^{-1} fresh weight)	Dehydrogenase (mg g^{-1} fresh weight)	Total soluble sugars (mg g^{-1} fresh weight)	Total phenols (µg g^{-1} fresh weight)
Control	0.52	124.07	1.40	17.50	49.00
Chilling (5^0C) for 4 weeks	1.46	405.62	4.00	28.00	17.90
Scarification with H_2SO_4 for 15 mins	1.41	384.62	3.40	26.50	23.00
Mechanical scarification followed by GA_3 100 ppm for 12 hrs	1.46	391.30	3.40	27.00	18.20
Mechanical scarification followed by GA_3 200 ppm for 12 hrs	1.38	378.42	3.60	26.00	23.01
KNO_3 1% for 24 hrs	1.72	432.34	4.30	28.00	15.01
KNO_3 1% for 48 hrs	1.75	434.25	4.70	32.00	15.00
GA_3 100 ppm for 48 hrs	1.75	464.32	5.70	32.90	15.08
GA_3 200 ppm for 48 hrs	1.89	507.26	6.00	33.01	14.90
Thiourea 0.5% for 24 hrs	1.55	405.62	4.30	29.00	18.00
Thiourea 0.5% for 48 hrs	1.65	424.71	4.60	30.00	15.09
C. $D_{0.05}$	0.001	0.064	0.025	0.004	0.001

and vigour of seeds by priming treatments i.e.KNO_3 1% for 48 hrs and thiourea 0.5% for 48 hrs. The improved germination and vigour of primed seeds may be attributed to the counteraction of free radicals and re- synthesis of membrane proteins (Saha 1990). Further, nitrogenous compounds in various forms, particularly nitrates (*e.g.*, KNO_3) play an important role in enhancing the physiological efficiency and influence the germination through changes in water relations. Other possible reason proposed for enhanced germination by KNO_3 is the shifting of the respiratory metabolism to the pentose phosphate pathway (Roberts and Smith 1977). Chilling at 5^0C for 4 weeks , scarification with H_2SO_4 for 15 mins , mechanical scarification followed by GA_3 100 ppm for 12 hrs and mechanical scarification followed by GA_3 200 ppm for 12 hrs were found beneficial in overcoming dormancy and improving the germination of seeds possibly by softening of thick and hard seed coat, resulting into water influx alongwith leaching of germination inhibitors. Similar findings were reported for another endangered medicinal plant species of north-western Himalayas i.e. Berberis aristata where scarification resulted in induction of germination and vigor (Thakur *et al.* 2006 a&b). Untreated seeds did not show germination which was substantiated by poor imbibition capacity , mobilization efficiency, alpha amylase, protease, dehydrogenase activities; lowest contents of total soluble sugars and highest total phenols thus reflecting the sub-optimal levels of metabolic activities due to dormancy.

GA_3 treated seeds also showed early onset of germination which might be due to early induction of protein alpha-amylase. Since, in seeds alpha-amylase is the enzyme which is most frequently credited with the initial attack on starch granules, resulting in the formation of simple sugars and ATP which is required for seed germination process. Reports of Trethewey and Smith (2000) were also in concurrence with our studies. The role of aleurone layer in forming the mRNA for protein (alpha-amylase) synthesis has been reported by Bewley and Black (1994). Gibberellins are known to promote the formation of amylases, proteases and dehydrogenases which degrade the reserve materials in seeds (Appleford and Lenton 1997). The enhanced activity of alpha-amylase during the pre-sowing treatments may be attributed to the higher imbibition capacity and mobilization efficiency during the above

treatments, activation of hydrolases resulting in increased breakdown of starch into soluble sugars which supports seed germination and vigourous seedling growth. This was also accompanied by lower phenol contents pointing out towards the decline in dormancy inducing substances. This is in concurrence to our findings which showed higher imbibition capacity during the above treatments resulting in increased contents of total soluble sugars which in turn is formed through enhanced alpha-amylase activity as also reported by Fincher (1989) and Lee and Kim (2000) who reported that increased alpha-amylase activity was associated with higher germination in coarse rice.

Present study thus, suggests that GA_3 100 or 200 ppm for 48 hrs and KNO_3 1% for 48 hrs were effective seed treatments and can be exploited to induce germination and vigor of *Rauvolfia serpentina* seeds.

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