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Research Article

OVERCOMING SEED DORMANCY OF *ACALYPHA INDICA* L. (EUPHORBIACEAE): AN IMPORTANT MEDICINAL PLANT

Santanu Gupta[1] and Abhijit Bandopadhyay[2]
[1, 2]Department of Botany, The University of Burdwan, West Bengal
**Author for Correspondence*

ABSTRACT
The seeds of *Acalypha indica* L., an important medicinal plant, are stubbornly dormant. Remarkable enhancement of germination (97.8%) has been achieved in combination with cold stratification (72 hours) and GA_3 (400ppm) treatment. Moreover, significant increase in Coefficient of velocity (24.8) coupled with noticeable decrease of T_{50} values (0.66 days) were recorded as a consequence of this treatment. This regimen of cold stratification with GA_3 obviously contributes to stimulate germination of seeds of *Acalypha indica* L. This protocol has potential to be utilized in overcoming the innate seed dormancy of different medicinally important plants for their propagation and conservation.

Key Words: *Cold Stratification, Acalypha indica L, Parts Per Million (ppm), Coefficient of Velocity (CV), Dormancy, GA_3*

INTRODUCTION
Seeds are excellent dispersal units which have emerged in the course of plant evolution. The chemical energy synthesized during photosynthesis accumulates in seed reserves' in multiple forms including carbohydrates proteins and lipids. Mobilization of these reserves is essential for the embryo to complete seedling establishment and to, begin a new life cycle (Nonogaki-2006). Seedling establishment is a critical stage in the life history of any plant species that relies on sexual reproduction for the persistence of its populations (Grubb, 1977; Harper, 1979 and Bu *et al.,* 2008). Variations in seed dispersal efficacy or germination percentage are often interpreted as reflecting adaptations to specific ecological conditions (Grime *et al.,* 1981 and Nishitani and Masuzawa, 1996)

Acalypha indica L. a small erect shrub commonly known as three- seeded mercury, is a well known medicinal plant belonging to the famiy Euphorbiaecae. This plant widely grows throughout the tropical parts of the world. However in India, it is quite commonly seen in shady places in and around the wasteland. A mature plant nearly reaches 60 cm in height with few ascending branches, leaves 3-5 cm long, ovate, flowers minute, crowded distally, scattered throughout the inflorescence, seeds small clustered together. This plant is highly popular in traditional Indian medicine, mostly effective against incipient phthisis, arterial hemorrhage, and also used as an anodyne, antihelmintic, emetic, cathartic expectorant. This plant has also got Nitric Oxide (NO) scavenging activity and is quite effective against complicated free radical related disease (Balakrishnan *et al.,* 2009). There are also reports on the anti-diabetic property of the plant extract on rats (Masih *et al.,* 2011). The leaf extracts *A. indica* L. in different organic solvents like acetone, chloroform etc have larvicidal activity against *Aedes aegypti*, *Anopheles stephensi,* and *Culex quinquefasciatus* **(**Kumar *et al.,* 2011**).** Mainly the roots, stems, leaves and flowers are used for medicinal purpose.

Seeds of *A. Indica* are difficult to germinate due to dormancy. Dormancy can be defined in multiple ways. Seed dormancy can be defined simply as inhibited germination of an intact viable seed to optimize the distribution of germination over time (Bewley and Black, 1983 and Hilhorst, 1995). Baskin and Baskin (1998) have reported that among 300 temperate herbaceous species, dormancy breaking and germination requirements are not phylogenetically constrained. Seed dormancy can be divided into two types: external and internal. Morpho-physiological dormancy belongs to the internal type of seed dormancy. According to the classification system proposed by Baskin and Baskin (1998; 2004), physiological dormancy is the most common form, found in seeds of gymnosperms and of all angiosperms. Previous research reports

from this laboratory suggest existence of remarkable physiological dormancy in *A. Indica* L. seeds (Sinha and Bandopadhyay, 2012).

Dormancy may be strong to weak, and the extent of dormancy present at any particular moment is referred to as the degree of dormancy. Dormancy patterns are similar for closely related taxa but may differ within a family, even between co-occurring species with similar life histories (Karlsson et al., 2006). Recent discoveries using *Arabidopsis thaliana* have provided additional information regarding the molecular and biochemical mechanisms involved in seed dormancy and germination. Genes like APETALLA 2(AP2) plays a key role in controlling seed mass/yield. Moreover roles of genes like HAIKU (IKU 2) and TRANSPARENT TESTA GLABRA 2 (TTG2) has been correlated with seed size through integument and endosperm (Nonogaki, 2006).

Seeds of most cultivated plant species lose their dormancy before or shortly after being separated from the mother plant, but in contrast, seeds of most wild shrub species have long seed dormancy (Bryant, 1996). Because seed dormancy is a nuisance in propagation programme, removing or breaking seed dormancy is a challenge for plant physiologists (Baskin and Baskin, 1998).

GA_3 is one of the most common plant hormone that has been suggested to break seed dormancy (Nadjafi *et al.,* 2006).Gibberellins and Abscisic acid are the hormones that control seed dormancy, where the first one act as a inducer and the second one as a inhibitor (Hilhorst and Karssen, 1992). Moist chilling or cold stratification has been widely used for breaking seed dormancy which increases germination percentage of most species (Sahoo, 2009). Seeds of many species of Apiaceae have morpho-physiological dormancy which can be removed through chilling (Baskin *et al.,* 1999 and Phillips, 2003).

Objective

Due to this inbuilt dormancy of seeds of *A. indica* L., the current paper focuses on this issue and proposes a simple protocol to tackle this problem. Moreover, the idea behind developing such a protocol is to generate a large number of disease-free plants via micropropagation using cotyledonary leaf as explants. This will be quite helpful for further biochemical analysis in future.

MATERIALS AND METHODS

Fresh seeds of *Acalypha indica* L. were collected from Burdwan and adjoining areas (Lat-23°15´N & Lon-87°51´E) in the month of June 2012. It was observed that this plant prefers to grow abundantly in the shady areas.

Germination tests were carried out immediately after collection and drying. Embryo-filled (viable) seed lots were surface sterilized using in 0.1% mercuric chloride ($HgCl_2$) for 1-2 minutes and then rinsed in distilled water. Some preliminary treatments were made to determine the duration of cold stratification and concentration of GA_3. From preliminary screening, three cold stratification treatments (24,48, &72 hours) and five concentration of GA_3 (100ppm, 200ppm ,300ppm, 400ppm, & 500ppm) were selected. For cold stratification, seeds were kept in a refrigerator at 4^0c- 6^0c for 24, 48, &72 hours. The hormone solutions were applied as pretreatment for 24 hours of imbibition immediately after 24, 48 &72 hours of cold stratification and then washed thoroughly in distilled water. Similar treatment was made using distilled water (D\W) as control. This resulted in 15 treatment sets which are named numerically from 1 to15. As a consequence 1 refers to control, 2 refer to 24 hrs cold stratification and 100 ppm GA_3 and so on (see Table 1).

The germination test was carried out in sterile petridishes of 9 mm in size placing a Whatman no. 1 filter paper on petridishes and moistened with distilled water. Each of cold stratified and GA_3 treated seed lots of every concentration was placed in 4 replicates with 25 seeds in each replicate. The petridishes were placed in a germination chamber in a photoperiod of 16:8 and a temperature of ($25^0C \pm 1^0C$) and relative humidity 65-70%. The seeds were allowed to germinate and data were recorded everyday over a period of one week. A seed was considered germinated when the tip of the radicle had grown free of the seed coat (Auld 1988). The results were recorded daily by counting the number of germinated seeds. The data were subjected to analysis of:

a**) Mean Germination percentage**=
$(No\ of\ germinated\ seeds)\ /\ (Total\ seeds\ in\ treatment)\times 100$
b) **Coefficient of velocity (CV)** = $(\sum Ni)\ /\ \ NiTi \times 100$ where N_i is the number of seeds germinated on i^{th}days T_i is the number of days from sowing (AOSA1983).
(Larger CV indicates high germination at a small germination time)
C) **Time to 50% germination (T_{50})** was calculated based on the following equation $\mathbf{T_{50}} = (ti + (N/2 - nj)(ti - tj))/(ni - nj)$ (Where N is the Final number of seeds germinated and ni and nj are the cumulative number of seeds that germinated by adjacent counts at time ti and tj where ni<N/2<nj) (AOSA 1991)

Statistical Analysis

The experiment was conducted to analyze the impact of different treatment sets (duration of cold stratification and concentration of GA_3) with three important seed germination parameters namely, mean germination percentage, coefficient of velocity and T_{50} values. The obtained data were statistically analyzed (using Microsoft Office Excel-2007) to find correlation among them. The data were also statistically analyzed by Analysis of Variance (ANOVA).

RESULTS AND DISCUSSION

The effect of different concentration of GA_3 and duration of cold stratification on seed germination percentage varied significantly under 16:8 photoperiods (Table 1). The germination percentage was considerably lower in control group (6.6%) in which no cold stratification or exogenous GA_3 was applied. The germination percentage showed an increasing trend with long exposure to cold stratification in combination to different analytical grades of GA_3, highest being at 72 hours cold stratification and 400 ppm GA_3 (97.8%). In 24 hours cold stratified seeds, the germination percentage increased with increasing concentration of GA_3, with maximum at 500 ppm (45.3%). Seeds cold stratified for 48 hours and imbibed in different grades of GA_3 showed higher germination percentage (highest at 500ppm -94.2%) in comparison to seeds stratified for 24 hours. Furthermore, 72 hours cold stratified seeds in combination with GA_3 showed the best result as mentioned above, in comparison to the two previous treatments

The coefficient of velocity (C.V.) of germination was found lowest at control set (10.47) that was deprived of cold stratification and exogenous GA_3 treatment. On increasing the duration of cold stratification and concentration of GA_3, the C.V. exhibited an enhancement, maximum being at 72 hours cold stratification and 400ppm GA_3(24.8). This indicates quick germination rate within a smaller duration of time. On the other hand, 24 hours cold stratified seeds combined with GA_3 showed lower C.V. values in comparison to 48 hours cold stratified and GA_3 treated seeds, where the highest C.V. was observed at 500 ppm GA_3 (20.2). In 72 hours cold stratified and GA_3 treated seeds, the C.V. was significantly higher in comparison to the other two treatment sets

The time to attain 50% germination (T_{50}) decreased drastically in cold stratified and GA_3 seeds in comparison to control (5.1 days) indicating less time to achieve the landmark. The T_{50} value decreased considerably in 72 hours cold stratified and 400 ppm GA_3 treated seeds (0.66 days) in comparison to 24 hours (lowest at 500 ppm: 2.9 days) and 48 hours (lowest at 500ppm: 1.12 days) cold stratified seeds. In Fig. 1, the regression analysis reveals a strong correlation (R^2=0.851) in mean germination percentage with respect to different treatment sets. A noticeable increase in mean germination percentage has taken place between two treatment sets i.e. 24 hrs of cold stratification and 500 ppm GA_3 & 48 cold stratification and 200 ppm GA_3 Similarly, (Fig. 2) coefficient of velocity reveals a steady correlation (R^2=0.850) with different treatment sets. As regards to T_{50} values (in days), the plot (Fig. 3) expectedly shows a negative correlation (R^2=0.898) with different treatment sets, because faster mean germination percentage is reflected by simultaneous decrease in corresponding T_{50} values. The ANOVA (Table 2) showed all the data (F value) were not significant (NS) at p=0.05 and p=0.01.The pattern of distribution of data sets in correlation analysis strongly indicate and support the effect of combined treatment of cold stratification and GA_3 concentration for promotion of germination of seeds of *A.indica*.

In preliminary screening it was found that only cold stratification or exogenous application of GA_3 gave no significant result (no seed germinated in any of the treatments or exhibited very low germination percentages). Thus it shows that both conditions contribute significantly to potentiate germination for seeds of *A. indica* L. The important role of the plant hormones gibberellins (GAs) in promoting seed germination is indicated by several observations. In plant species such as *Arabidopsis* and tomato, the strong alleles of GA-deficient mutants are unable to germinate without exogenous GAs (Koornneef and Veen, 1980). A germination-promoting role for GAs has also been deduced from their ability to overcome germination constraints that exist in seeds requiring after-ripening (Metzger, 1983). In *Swertia chirayita*, pre–sowing treatments of GA_3 (50-350µM) gave most effective stimulation by increasing germination percentage (96.7%) and lowering MGT (14.16) over the control (Pradhan and Badola, 2010). Cumulative percent germination increased three fold over the control value when seeds were stratified for 2 weeks or more with greater CV values for hop hornbeam copperleaf (*Acalypha ostrylifolia* L.), indicating a faster germination rate with longer cold treatment over 1- to 3-wk interval (Horak *et al.*, 1998). In *Ferula assafoetida* L. maximum germination was obtained at combination treatment of cold stratification (60 days) with 2000 ppm of GA_3 (91.66%) and minimum germination was at soaking in running water and control treatments (Zare *et al.*, 2011).Similar results were obtained in *Ferula assafoetida* L. combining BAP (25mg/L) and 28 days of chilling, where 90 % germination was obtained (Otroshy *et al.*, 2009).

Table 1: Mean germination percentage (%), Coefficient of velocity (C.V.) and Time to 50% germination (T50) of A.indica L.seeds in different treatment series. (Mean± standard deviation)

Duration of Cold Stratification (Hours)	Concentration of GA_3 (ppm)	Mean germination percentage (%)	Coefficient of velocity (C.V.)	Time to 50% germination (T_{50}) in days
0(Control)	0 (1)***	6.6±0.77	10.4±0.14	5.1±0.12
	100 (2)	18.66±0.38	14.4±0.25	4.5±0.16
	200 (3)	22.66±0.50	15.1±0.36	4.1±0.29
24	300 (4)	30.6±0.97	15.5±0.29	3.6±0.14
	400 (5)	38.33±1.43	16.3±0.23	3.2±0.11
	500 (6)	45.33±0.38	17.1±0.31	2.9±0.18
	100 (7)	82±1.73	17.7±0.17	1.8±0.12
	200 (8)	86.4±1.25	18.5±0.23	1.7±0.11
48	300 (9)	88.66±0..86	19.5±0.17	1.5±0.11
	400 (10)	90.2±0.91	20.1±0.63	1.13±0.09
	500 (11)	94.2±1.76	20.2±0.47	1.10±0..08
	100 (12)	84.44±0.47	17.3±0.25	1.3±0.14
72	200 (13)	91.11±1.12	20.7±0..11	1.18±0.16
	300 (14)	95.6±0.42	22.5±0.46	0.96±0.05
	400 (15)	97.8±0.45	24.8±0.49	0.66±.14
	500	Nd**	Nd**	Nd**

***Nd- not determined*
**** In parenthesis 1, 2 and 3 etc are the treatment sets that are named numerically*

Table 2: Analysis of Variance (ANOVA) in mean germination percentage, Coefficient of velocity &T_{50} values among different treatment sets for germination of seeds of *A.indica*. at p =0.05 & p=0.01

Parameter	Sum of Squares	df	Mean Square	F	Sig.
Mean germination percentage	61985.911	14	4427.565	4332.727	.000
Coefficient of velocity	701.949	14	50.139	429.765	.000
T_{50}	120.307	14	8.593	398.262	.000

In *Pedicularis olympica*, the highest germination rate was found in the seeds treated with 250 ppm GA_3; 64% of these seeds germinated when treated with moist chilling and incubated in the dark, while 75% germinated under 12/12 hrs photoperiod condition (Kirmizi *et al.*, 2010).

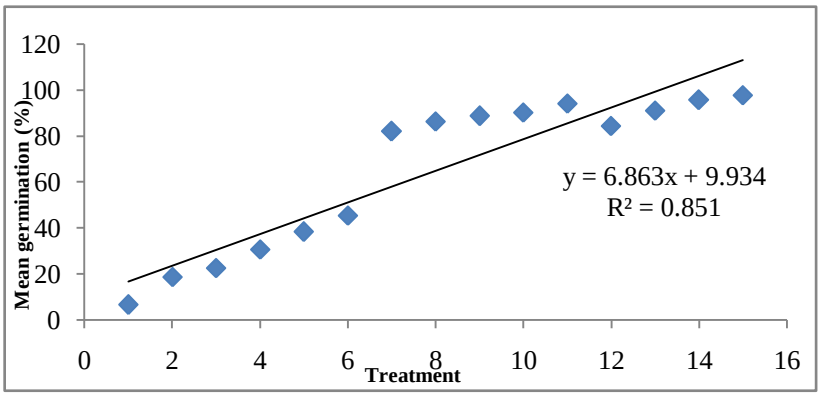

Figure 1: Plot showing correlation of different sets with mean germination percentage

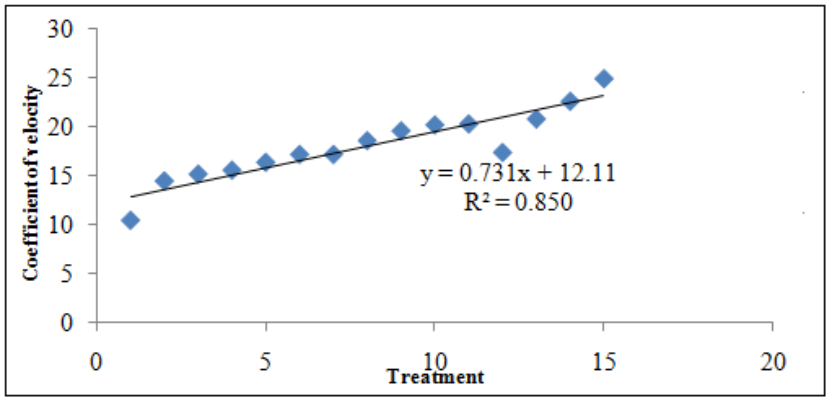

Figure 2: Plot showing correlation of different sets with coefficient of velocity

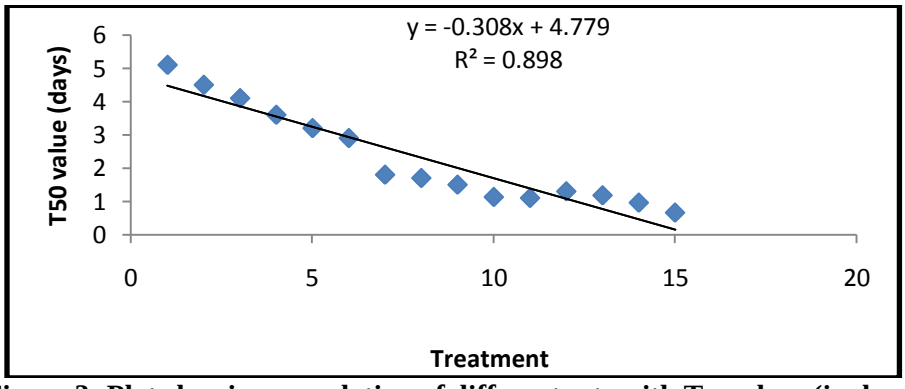

Figure 3: Plot showing correlation of different sets with T_{50} values (in days)

Dormancy is one of the most important physiological properties that are controlled by ABA/GA_3 ratio. At high levels of ABA and low levels of GA_3, the embryo remains dormant and with a reversed ABA: GA_3 ratio, the intensity of embryo's dormancy is reduced (Finch – Savage and Leubner-Metzger, 2006). Cold stratification might act simply to lower the rate of enzymatic reactions taking place in the seed, and might cause differential changes in enzyme concentrations or in enzyme production (Bewley and Black, 1994).

The main reason to explain the effect of chilling on breaking seed dormancy is enhancing internal GA_3 accumulation (Phillips *et al.*, 2003). GA_3 is suggested to break primary dormancy and stimulate germination (Eastmond and Jones, 2005). In *Pedicularis olympica* seeds, GA_3 enhances the degradation of food reserves in endosperm by stimulating hydrolytic enzymes (Da Silva *et al.*, 2005). Thus if chilling enhances the accumulation of GA_3, then application of exogeneous GA_3 causes more and quicker germination, as noticed in our experiment. Seeds of strawberry tree (*Arbutus andrachne* L.) stratified at 4°C for 12 weeks had 86% germination (Karam and Al-Salem, 2001). Stratification had a significant effect on seed germination of black mulberry (*Morus nigra* L.). Non-stratified seeds gave only 33% germination, whereas seeds stratified for 100 days gave 88% germination. Increasing the duration of stratification from 0 to 100 days resulted in up to a 164% increase in germination. The combined treatment of 250 mg/l GA_3 and 100 days of stratification yielded 96% germination of seeds (Koyuncu, 2005). Koyuncu and Sesli (2000) reported that 125 days of stratification had a significant effect on the germination percentage of *Juglans regia* L. nuts.

In this paper, it is evident that there is a significant increase of all the parameters of seed germination in cold stratified seeds treated with GA_3 as compared to that of control. It may be concluded that, the combined regimen of cold stratification and exogenous GA_3 can effectively break the inbuilt dormancy of seeds of *Acalypha indica* L.

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