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OPTIMISATION OF SEED GERMINATION AND SEEDLING EMERGENCE OF *SESBANIA CANNABINA* (RETZ.) POIR.

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Abstract

This study aims to optimise the condition for seed germination and seedling emergence of *Sesbania cannabina* (Retz.) Poir. for a phytoremediation study. In this experiment, seed germination was carried out using two growth media: Murashige and Skoog basal medium (MS); and Whatman Grad 1 filter paper (FP), using the top of media or top of the paper method under three different photoperiods. Seeds were pre-treated for different lengths of time with various concentrations of hydrogen peroxide (H_2O_2) (v/v) multiple hot water treatments. Results showed that seeds pre-treated with H_2O_2 (6% v/v) for 5 minutes and primed with 65°C water for 5 minutes were considered as the ideal pre-treatment condition. Different photoperiods and media used in germination in this experiment do not significantly affect seed germination. The optimum condition for seed germination at 27.5°C ± 2.5°C and relative humidity of ~ 75% for 5 days. Seed emergence in soil and compost was significantly affected by the burial depth and bulk density of the media, with the highest (98% ± 1) seed emergence observed at 1 cm depth for soil and compost, and decreasing with increased burial depth. This ideal condition will help in further studies related to plant growth and phytoremediation of *S. cannabina.*

Introduction

The accumulation of heavy metals (HM) in the biosphere due to anthropogenic activities has become a widespread problem (Zwolak *et al.* 2019, Awa and Hadibarata 2020, Vardhan *et al.* 2019). To date, there has been substantial research in the field of soil pollution, its effect on plants and phytoremediation, including the effects on seed germination and early stages of plant growth (Kuriakose and Prasad 2008, Munzuroglu and Geckil 2002). Phenotypic and morphological changes occur in response to various biotic and abiotic stressors (e.g. heavy metals) during seed germination, which is very important for the plant life cycle (Wojtyla *et al.* 2016). The actual effect of contaminants on seed germination is difficult to assess until 100%, or maximum seed germination, is achieved before toxicological studies. Some research has failed to state the maximum germination condition in their research, providing an important limitation to the interpretation of these studies (i.e Zhi *et al.* 2015, Guterresa *et al.* 2019, Sahoo *et al.* 2018).

Numerous environmental factors affect the germination of wetland species, including daytime temperature variations, water availability, oxygen, flooding and shallow sediment cover (Lorenzen *et al.* 2000, Webb *et al.* 2009). For some types of seeds, seed cover hinder water penetrate and preventing the embryo from growing (Shreelalitha *et al.* 2015, Chanda *et al.* 2017). Many plant species have been studied for phytoremediation of heavy metals and it is crucial to understand the seed germination condition before phytoremediation studies. It has been reported that *Sesbania* seeds of Fabaceae or Leguminosae family have a seed coat that prevents imbibition and thus hinders germination (Guppy 1912). There are several ways to increase seed germination rate in the presence of a seed coat and among the most important are (a) temperature (e.g. hot water treatment) (Iqbal *et al.* 2019) (b) pre-treatment with Polyethylene Glycol (PEG 6000) (Muscolo *et al.* 2014) (c) use of beneficial fungi (*Trichoderma harzianum*) (Bharath *et al.* 2005) (d) seed

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disinfection (e.g NaClO or H_2O_2) (Iqbal *et al.* 2019, Chigbo and Batty 2013) and (e) use of Gibralic Acid (GA3) (Kołodziejek *et al.* 2017; Lawes and Anderson 1980). Several techniques have been applied to increase the germination rate of legume species seeds, such as hot water treatment and physical or acid scarification (Dan and Brix 2007).

S. cannabina, an annual shrub, is commonly found in China, the Indian sub-continent, southeast Asia, Papua New Guinea, Australia and the South Pacific Islands (Sarwar *et al.* 2015). Summer environments are suitable for rapid growth (up to 3.5 m) and development; however, the plant can grow in spring and autumn (Rao and Gill 1995, Sarwar *et al*. 2015). Each mature plant can produce around 1,200 pods, which contain about 24 thousand more or less dark green to brown, rod-like seeds (Rao and Gill 1995; Sarwar *et al*. 2015). The present study aimed to determine suitable conditions for maximum germination of the seeds of *S. cannabina.* To achieve maximum germination and seedling emergence, we have considered the following experiment steps (1) identification of the appropriate dose of disinfectants (H_2O_2) and ambient temperature for hot water treatment of seed before germination, (2) suitable media for germination, (3) ambient air temperature and photoperiod for seed germination, and (4) seedling emergence from different burial depth of seeds.

Materials and Methods

For use in all experiments, seeds of *S. cannabina* were collected in 2018 from Shobuz Biz Bhandar, Bangladesh, a locally reputed seed-selling company and stored under dry conditions at 4 °C temperature (Webb *et al*. 2009) for six months before use (Figs 1 A and 1 B).

Seed selection was performed by examination under a dissecting microscope, and deteriorated seeds (e.g., dirty or void seeds) were rejected. Initially, selected seeds were then surface sterilised and treated with hot water. After treatment, seeds that appeared to be fully imbibed (Fig 1. B) were selected for the germination experiment.

Fig. 1. Images of *S. cannabina* seed 1(A) healthy seeds before treatment, 1(B) after imbibition of seeds treated with hot water (65°C).

To determine the effectiveness of disinfectant on seed germination, in this experiment, healthy seeds were surface sterilized (soaked) in an orbital shaker (200 RPM, 5 min) with different concentrations (1, 2, 3, 4, 5, 6, 7 and 8%) of hydrogen peroxide (v/v) (Chigbo and Batty 2013, Wojtyla *et al.* 2016). Following treatment, seeds were rinsed with de-ionised water and transferred to a petri dish. Five replicates of each treatment were prepared with 10 seeds per dish. These were

incubated for five days at an air temperature of 25°C. After five days, a spot check for microbial growth was performed under the dissecting microscope. If any microbial growth was evident, this was considered positive (+) and where there was no visible microbial growth, negative (-). Minimum concentrations that showed maximum germination with negative results were considered a suitable disinfectant dose.

After surface-sterilisation (at a concentration which maximum germination and no microbial growth), seeds were primed with different temperatures of hot de-ionised water temperatures of 50 , 55 , 60 , 65 , 70, 75, 80, 85, 90, 95 and 100°C for 5 min. After treatment, seeds were placed on double-layer Whatman® 1 filter paper (FP). For each hot water treatment, 10 seeds were placed in each petri dish (9 cm diameter) on a growth medium (e.g., filter paper and MS media) with 6 replicates of each treatment.

A wide variety of growth media have been used within germination experiments, including Murashige and Skoog basal medium (MS) (Siddiqui *et al.* 2014) and filter paper (moistened with 5 ml deionized water) (Chigbo and Batty 2013) and Bacto agar (Bae *et al.* 2016). In this experiment, modified Murashige and Skoog basal medium (MS) and filter paper (FP) (Whatman Grad 1 filter) were compared for seed germination. Whatman Grade 1 filter paper (FP) meets the ISTA (International seed testing association) requirement (Healthcare, n.d.). MS medium is also used in phytoremediation studies, especially for germination and seedling growth (Santiago-Cruz *et al*. 2014, Lusa *et al*., 2019). Both media did not contain any persistent, bio-cumulative or toxic compounds for plant growth (Buendía-González *et al*. 2010).

To determine how growth medium affects seed germination, 10 seeds were placed petri dish (in 9-cm-diameter, six replicates) on the growth medium (double layer of Whatman No. 1 filter paper (FP) and modified Murashige and Skoog basal medium (MS)). The growth media were then soaked with 5.0 ml of de-ionised water (Milli-Q® Gradient A10^{TM}), the petri dishes were wrapped with para-film to avoid evaporation and placed in a vitopod® propagator (temperature controlled, fixed at 28 °C ± 1 °C). In this experiment, MS was modified by adding sucrose and agar, modified MS medium containing 4.4 gm MS, 30 gm sucrose and 8 gm nutrient agar per litre of medium (Buendía-González *et al*. 2010).

To understand the effect of temperature and photoperiod on seed germination, seeds were incubated at seven different fixed temperatures (5, 10, 15, 20, 25, 30 and 35 °C) under three different light treatments, 1. darkness (24 h), 2. light (24 h) and 3. photoperiod (12/12-h). Petri dishes were covered with carbon paper to create darkened conditions for germination. All seeds were pre-treated with H_2O_2 and hot water (65°C).

Borosilicate glass cylinders (42 mm diameter x 310 mm height) with no drainage holes were filled to approximately 280 mm depth with growth medium [soil (~360 gm) or compost (~120 gm)] and covered with dark paper. Two growth media were used in this experiment. The first was a sandy loam soil (supplied by Singletons Nurseries, UK) with a pH of 7.3, containing 2.1 % organic matter 62, 87, and 412 kg ha^{-1} of N (nitrogen), P (phosphorus), and K (potassium) respectively. The second was compost (supplied by Singletons Nurseries, UK) with a pH of 5.3-5.8, containing ≥ 57 ± 2 % organic matter and 204, 104, and 339 kg ha^{-1} N,P,), and K , respectively.

Before use, the soil/compost was autoclaved (to avoid contamination) and sieved with a 2-mm mesh net. Two seeds were placed at different depths for each treatment (with three replicates) and then covered with soil/compost to form depths of 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 cm and irrigated with di ionized water. A depth level of 0 cm (top of soil/compost) was excluded due to the risk of inadequate seed-soil/compost interaction and lower water uptake (Messersmith

et al. 2000) and predation by pests (Chauhan *et al.* 2012). We consider seedling emergence while the shoots appear at the soil surface and were recorded every 24h from sowing up to 7 days.

Germination percentage (TG_{hour})(at 12-hour intervals) was calculated using the formula (Bae *et al*., 2016):

$$TG_{hour} = \frac{\text{Number of germinated seeds at fixed hour}}{\text{Total number of seeds}} \times 100$$

Pearson Correlation (2-tailed) (correlation is significant at the 0.01 level) and One-way Analysis of Variance was carried out with SPSS (v 25). We checked normality and homogeneity of variance assumptions before ANOVA. When a significant ($p < 0.05$) difference was observed between treatments, multiple comparisons were made using the Tukey post-hoc test.

Results and Discussion

The results showed that ≥ 6 % H_2O_2 (v/v) concentration was most suitable for the disinfection of *S. cannabina* seeds (table 1). A higher concentration (≥ 30 % v/v) of H_2O_2 has the capacity to damage tissues (Public Health England 2009), and for that reason, 6 % H_2O_2 is considered the most suitable concentration for seed disinfectants.

Table 1. Effect of different concentrations of H_2O_2 (where (+) means microbial growth and (–) is no microbial growth).

Dose (v/v)	Petri dish 1	Petri dish 2	Petri dish 3	Petri dish 4	Petri dish 5
1 %	+	+	+	+	+
2 %	+	+	+	+	+
3 %	+	+	+	+	+
4 %	+	+	-	+	-
5 %	-	-	+	-	-
6 %	-	-	-	-	-
7 %	-	-	-	-	-
8 %	-	-	-	-	-

Hydrogen peroxide was previously recognised as a harmful chemical can damage cell or cell viability. Many studies have focused on the function of hydrogen peroxide in seed germination (Wojtyla *et al*. 2016; Barba-Espín *et al*. 2012); but the actual function of this molecule remains unknown. The main function of H_2O_2 in seed germination is recognised as disinfection of the seed and as a signalling molecule for germination (Barba-Espín *et al*. 2012).

In this section, after H_2O_2 (6 %, v/v, 5 min) treatment, seeds were primed with different water temperatures and allowed to germinate in the germination chamber. Within 60 hrs, the maximum germination was achieved under conditions of 65 °C (98.2 ± 1 %) (pre-treatment for 5 min) (Fig. 2).

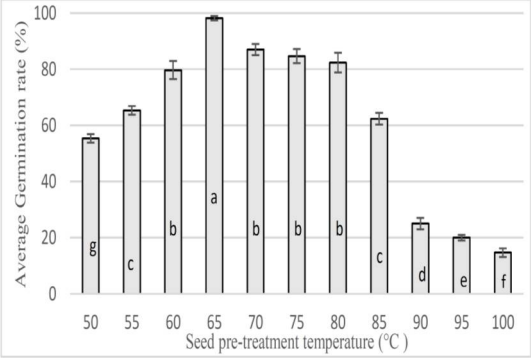

Fig. 2. Effect of seed pre-treatment with hot water (for 5 min) on germination seeds at day 5. Error bars are standard error (n = 5). Identical letters indicate no significant difference, as determined by Tukey's LSD ($p \leq 0.001$).

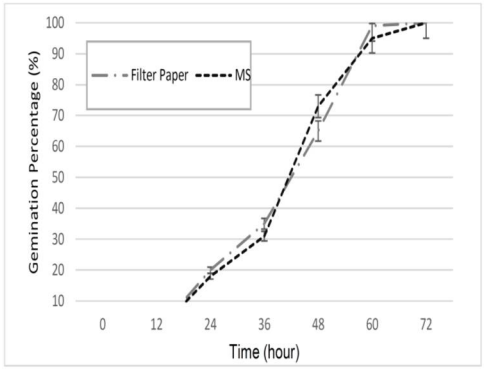

Fig. 3. Seed germination on two different growth media. Error bars are standard error (n = 5).

Due to low water permeability, the hard seed coat of *S. cannabina* makes the seed physically dormant, and this morphological characteristic makes these seeds resilient to various stressors (Veasey *et al.* 2000). The low temperature of the water is inadequate to soften the seed coat, while high temperature has a lethal impact on seed germination (Iqbal *et al.* 2019). Dan and Brix (2007) showed that 70 ºC hot water pre-treatment produced a higher *S. sesban* seed germination rate than pre-treatment with 60 ºC water or 98 % H_2SO_4. In another study, seeds of *S. sesban* were first soaked in water at 80 °C for 8 min to achieve maximum germination (Wang and Hanson, 2008).

Iqbal *et al.* (2019) achieved 95 % ± 1 seed germination for *S. cannabina* by soaking seeds with sodium hypochlorite (NaClO) (1% v/v) for 1 min, followed by pre-treatment with boiling water (100 ± 2°C). In our experiment seeds treated with H_2O_2 (6 %, v/v) showed higher germination percentages than sodium hypochlorite (Iqbal *et al.* 2019). In addition to that, the concentration used in our experiment has been found in many domestic (chlorine-free) bleach products and is more environmentally friendly than sodium hypochlorite (Public Health England 2009, 2015, SCHER 2008). 65 ºC hot water (for 5 min) was found to be the best suitable pre-treatment temperature for seed germination. In this experiment, we observed maximum germination (in 5 days) with a combination of pre-treated with H_2O_2 (6 %, v/v) and 65 °C hot water (for 5 min). Again, no significant difference ($p > 0.05$) was observed on seed germination rate between two different media (Fig. 3)

Seed germination on different temperatures (constant incubation temperatures), the results showed that temperature had a significant effect on germination ($p \leq 0.005$), and also observed no significant effect of photoperiod on germination ($p > 0.05$) in each constant incubation temperature. In addition, only 0-4% germination percentages were observed in 12/12 hrs photoperiod compared to day and night photoperiod thus, we can conclude that the seeds demonstrated a neutral photoblastic response (Fig. 4) where seeds can germinate in with or without light. Research has previously shown that dark and light conditions do not affect germination for seeds of *S. sesban* (Dan and Brix 2007, Graaff and Staden 1984). This may allow germination from greater burial depths (Fig. 5).

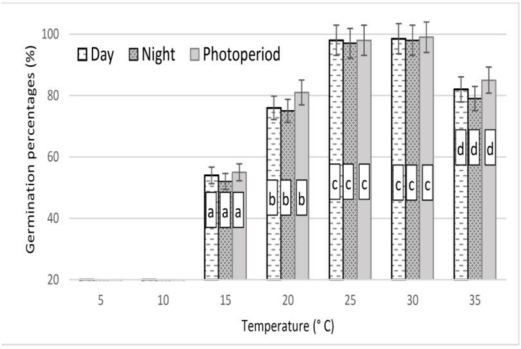

Fig. 4. Effect of different (constant) temperatures and different photo-period on seed germination of for 5 d. Error bars are standard error (n = 5). Identical letters indicate no significant difference, as determined by Tukey's LSD ($p \leq 0.001$).

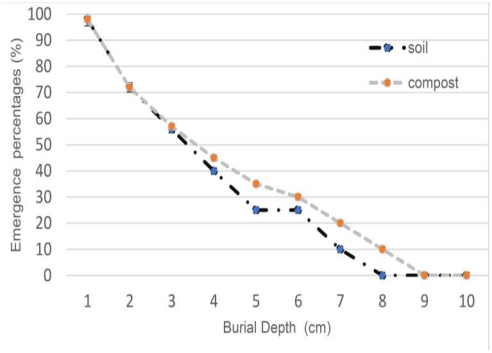

Fig. 5. Seed emergence results of *S. cannabina* grown on soil and compost in a glasshouse at different depths, 28 C for 7 d.

The highest germination was recorded at temperatures between 25 and 30°C, and germination percentages declined slowly as temperatures increased (> 30°C) or decreased (< 25°C) (Fig. 4). However, we observed no germination below 10°C. Enzyme activity and hormone synthesis of seeds (during germination) impacted by growth chamber or room temperature (Baskin and Baskin 2014). Thus, temperature (germination chamber or air) has a very high impact on germination. Research on *S. sesban* showed that the highest germination was observed at temperatures between 30 and 37ºC, but germination stopped below 13ºC or above 45ºC (Dan and Brix 2007). In a similar study, Iqbal *et al.* (2019) observed the highest germination of *S. cannabina* seeds (87 %) at 32°C. *S. cannabina* seeds exhibit germination capacity in a wide range of temperatures, indicating that they have high adaptability and can germinate throughout the year in tropical countries.

We observed a significant impact of varying burial depths on the seedling emergence of *S. cannabina* ($p < 0.001$) (Fig. 5), and the result showed that with an increase in burial depth seedling emergence rate declined. Comparing the two-growth media, the seed grown in compost exhibited more potential towards germination through different burial depths and recorded 9% higher germination than the seed germinated in soil. For both compost and soil, we observed maximum emergence (98 ± 1%) for seeds buried under 1 cm depth, but with an increase in burial depth, seed germination and seedling emergence varied between the two media.

In this experiment, seeds buried ≥ 10 cm could not emerge within 7 days. We also observed that *S. cannabina* had the capacity to emerge from soil (≤ 7 cm) and compost (≤ 8 cm) within 7 days. Similar studies related to seedling emergence also show that seedling emergence had a negative correlation with seed burial depth (Mennan and Ngouajio 2006, Önen *et al*. 2018, Zhao *et al.* 2018). Stored food (carbohydrate reserve) within the seeds allows seeds to grow in dark conditions (Mennan and Ngouajio 2006), but *S. cannabina* seeds have a moderate reserve compared to other plant species (Chanda *et al.* 2017), thus allowing the plant to grow from the deep burial (≤ 9cm).

Conclusion

Identifying viable seeds for assessing the phototoxicity of contaminants for germination studies is crucial. In summary, the results of this study demonstrate that *S. cannabina* can germinate and emerge within diverse environmental conditions. Treatment with 6% (v/v) H_2O_2 (5

min) and 65 °C hot water (5min), allowed seeds of *S. cannabina* to germinate rapidly under growth conditions of 25 to 30°C. *S. cannabina* seed can emerge from burial depth in the soil (up to 8 cm) and compost (up to 9 cm) indicates that these seeds did not require any unique technique for cultivation.

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