Evaluation of potential seed coating formulation with *Bradyrhizobium japonicum* on soybean (*Glycine max* L.) seeds

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Abstract: The seed coating technique can be used as a potential biofertilizer delivery strategy. This study sought to formulate a low-cost potential seed coating mixture for soybean (*Glycine max* L.) seeds, applied with *Bradyrhizobium japonicum* and assess the viability of the bacterium on the seed coat during storage. Seed coating mixture was formulated by using xanthan gum and biochar as the binder and the filler respectively in the ratio of 80g:20g. The formula was further checked with three different xanthan gum concentrations (0.25%, 0.5% and 1%) each with and without sucrose (1%W/V). The selected coating mixture was inoculated with *Bradyrhizobium japonicum* (3×10^8 CFU/ml) and tested after 20 days of storage at two different temperatures (4℃ and 30℃) for *Bradyrhizobium* survivability and seed germinability. The germination percentage was significantly higher ($p<0.05$) in both coated and non-coated seeds stored at 4℃ compared to 30℃. Germination percentages of non-coated seeds were higher under both temperatures. Initially, the survivability of the bacterial population after seed application was around 1×10^7 CFU/ml in the seed coating but significantly dropped after three days in both temperatures. At the end of the 15th day the survivability of the inoculum was maintained to over 7×10^5 CFU/ml under 4℃. The formulated seed coating is effective for its transient capacity in maintaining the viability of *Bradyrhizobium japonicum* at lower temperatures. This can be developed as a potential biofertilizer to hold and later deliver to the field soil via seeds.

Keywords: *Biochar, biofertilizers, legumes, seed coat, seed germination, xanthan gum*

1. INTRODUCTION

Although it has a beneficial effect of increasing crop yield, excessive use of inorganic fertilizers causes severe damage to the ecosystem. It critically damages the soil quality (Chandini *et al*. 2019; Verma *et al*. 2012; Guo *et al*. 2010), and declines the soil microbial community (Geisseler and Scow 2014). The accumulation of heavy metals in the fields and their loss due to leaching, together with eutrophication are considered as the other significant problems. Apart from that, crops, grown in soil contaminated with heavy metals, contain high concentrations of those elements that are not suitable for consumption (Alengebawy *et al.* 2021). Therefore, in searching for an alternative solution for sustainable agriculture, one of the promising approaches would be the use of beneficial microorganisms as biofertilizers and biopesticides (O'Callaghan 2016).

Biofertilizers are substances containing living microorganisms, which can promote plant growth by supplying essential nutrients to the host plant. They can decompose and mineralize organic matter, thus enhancing nutrient mobilization (Rashid *et al.* 2016) and are capable of maximizing soil water holding capacity, buffering the soil aggregates from toxic substances by decomposing them etc. (Rashid *et al.* 2016). Apart from that, biofertilizers can restrict the nitrogen (N) and phosphorus (P) leaching loss from soil by slowly releasing nutrients from organic matter. The presence of beneficial microorganisms in biofertilizer-applied soils are reported to suppress soil-borne diseases, plant diseases, and parasite and pest attacks (Mabrouk *et al.* 2018; Pathma and Sakthivel 2012). As diazotrophic bacteria that are capable of fixing atmospheric nitrogen and fulfilling the nitrogen needs of plants, they represent a crucial role in biofertilizers. (Mabrouk *et al.* 2018; Mia and

Shamsuddin 2010; Poonia 2011). For legume cultivation, crop-specific *Rhizobium* spp. are being used as a biofertilizer worldwide through the establishment of an effective symbiotic relationship between the legume crop and the *Rhizobium* spp. (Mia and Shamsuddin 2010; Zahran 1999).

Rhizobium spp. reside in the legume roots inside a special structure called nodules. Within these structures, specific *Rhizobium* spp. can fix atmospheric nitrogen inside the effective nodules of the host plant. The formation of legume root nodules is facilitated by the root exudates of the legume root, and rhizobia in the soil reaches the legume roots by sensing those chemical substances of the root exudates (Via *et al.* 2016). Other than atmospheric nitrogen fixation, *Rhizobium* spp. can solubilize phosphorus compounds, and produce enzymes, phytohormones, and siderophores (Mabrouk *et al.* 2018; Rashid *et al.* 2019). Further, they are capable of producing exopolysaccharides which are helpful in soil aggregation and establishing symbiotic relationships. As legume plants are highly cultivated worldwide as food crops, instead of using chemical fertilizers, the use of specific *Rhizobium* spp. to improve crop yield might be a cost-effective and environmentally friendly strategy (Samarakoon and Yapa 2019). Therefore, it was evident that specific microbial inoculants can be applied to enhance the soil quality of agricultural fields. Currently, these inoculants are directly applied to the field or applied along with the seeds of legume crops differently. Each method has its pros and cons, and the researchers are trying to find better seed inoculation processes which maintain higher inoculum quality after seed application and are less costly (Deaker *et al.* 2004).

An application of an exogenous material on the natural seed coat is called seed coating. There are three main seed-coating methods; seed pelleting, encrusting and film coating (Pedrini *et al*. 2017). Most of the time, these methods are used to improve the physical characteristics of seeds (Avelar *et al.* 2012). Seed pelleting is often used to sort seeds through seed size and weight to facilitate handling (Halmer 2006). Film coating technology has been brought into the light by the pharmaceutical and food industries which are widely accepted by the seed coating technology (Accinelli *et al.* 2016). In this method, a very thin layer of polymeric substance is applied and along with this usually, fungicides, insecticides, herbicides, and pigments are applied. Hence, seed coating technology can be used to deliver other important substances which are essential to the plant such as nutrient amendments, growth hormones etc. (Wiatrak, 2013). The studies which have been done on delivering macronutrients to the field via seed coat have shown positive results (Peltonen-Sainio *et al.* 2006), but there is a possibility of nutrients-induced osmotic stress (Scott 1989). On the other hand, delivering micronutrients via seed coat (Adhikari *et al.* 2016; Hara 2013; Rehman and Farooq 2013; Wiatrak 2013) to treat soil deficiencies of microelements has been shown promising results (Farooq *et al.* 2012).

For effective delivery of beneficial microorganisms to the field with the seed coat, the seed coat must be able to stabilize the microbial population, protect the microbial population from desiccation during storage and after sowing, aid in delivering microbial population to the field or soil and finally to enhance their functionality in situ (O'Callaghan 2016). Delivery of associating rhizobacteria to the field via seed coating has been done by using spore-forming bacteria and biocontrol fungi (Accinelli *et al*. 2016; Accinelli *et al*. 2018). Nevertheless, the incorporation of non-spore forming bacteria and maintaining their survivability is challenging and need to be explored.

In seed coating process, there are three main substances; binder, filler, and active ingredient (Pedrini *et al*. 2017). A binder is a substance which has adhesive properties and, thus, maintains the structural integrity of the seed coat. Usually, a synthetic polymer is used by

seed companies. However, many studies have been done by using various substances as binders such as liquid bioplastics (Accinelli *et al*. 2016; Accinelli *et al.* 2018), alginate (Chhetri *et al*. 2019; Sarrocco *et al*. 2004), polyethylene glycol (Chandrika *et al*. 2019), gum Arabic (Dawar *et al*. 2008), polysaccharide pel gel (Ugoji *et al.* 2006), carboxy methyl cellulose (Zhou *et al*. 2017), methylcellulose (Lopisso *et al*. 2017) and xanthan gum (Amirkhani *et al.* 2019). Among these several binders it has been reported that xanthan gum and gum Arabic can be used as both a binder and an efficient delivering agent of beneficial microorganisms (Jambhulkar *et al.* 2016). Xanthan gum is a biopolymer which is widely used in the food industry as a thickening and stabilizing agent produced by the bacterium *Xanthomonas campestris* (Amirkhani *et al*. 2019). Previous studies showed that there was a better survival of beneficial microorganisms when inoculated into xanthan gum (Jung *et al*. 1982) but the feasibility of the application of xanthan gum along with beneficial microorganisms is not clearly understood.

Fillers are inert, powdery materials which are used to increase seed size and weight. Common fillers which have been used in studies so far are, peat (Hameeda *et al*. 2010), biochar (Williams *et al*. 2016), lime (Hartley *et al*. 2004), chitosan (Chandrika *et al*. 2019) and talc (Berninger *et al*. 2016). Biochar is commonly used in agricultural practices as a soil amendment which helps to improve soil quality and plant growth (Jayawardana and Yapa 2018). As it is a stable form of carbon, microorganisms cannot oxidize it. Biochar possesses a highly porous structure, hence having a large surface area. As its surface is highly negatively charged, it can absorb nutrients and gradually release them into the soil environment (Liang *et al*. 2006). It also has a high-water retention capacity thereby conserving water (Petter and Madari 2012). Most importantly, the porous structure and the enormous surface area provide a suitable microhabitat for soil bacteria and their population growth. Owing to this quality of biochar, it can be used in the seed coating process as a filler, which facilitates the effective delivery of a beneficial rhizobacterial inoculants to the field.

The scope of this study was to design a low-cost artificial seed coat using xanthan gum and biochar to hold and deliver *Bradyrhizobium japonicum* to the soil via soybean seeds. The efficiency and effectiveness of the seed coat were determined by comparing *in vitro* germination and microbial counts with the non-coated seeds.

2. MATERIALS AND METHODS

2.1 Seed coat formulation – Experiment 1

The following test was conducted to ascertain a suitable seed coating mixture ratio by mixing binder as xanthan gum and filler as biochar at different ratios. First, to obtain 1% of the xanthan gum mixture, 1g of xanthan gum powder was added slowly into a beaker filled with warm (70 °C) 100 ml of sterilized distilled water and thoroughly mixed with an electric hand mixer (Panasonic-MK-GH1) for about 10-15 minutes.

Next, total of five mixtures were prepared by mixing different ratios of xanthan gum (from 1% of xanthan gum mixture) and powdered biochar (80g:20g, 60g:40g, 50g:50g, 40g:60g, 20g:80g), i.e., 80 g of xanthan gum mixture was retrieved after allowing to cool at room temperature and thoroughly mixed with 20 g of finely ground, sieved, and sterilized biochar to make xanthan gum: biochar (80g:20g) mixture. The mixture was tested for its' initial seed coating applicability such as the percentage of seed surface coverage by the coating. For the coating process, soybean seeds were obtained from a farmer in Galenbindunuwewa, North Central Province, Sri Lanka. According to the results of the seed coating applicability test, a suitable seed coating mixture (xanthan gum and biochar) ratio (called mixture ratio A) was selected for further seed coating formulation.

2.2 Seed coat formulation – Experiment 2

As xanthan gum solution is a highly viscous, even smaller concentrations of xanthan gum would interfere with the emergence of the radicle although performs well with the inoculum. Hence, the three different xanthan gum concentrations (0.25%, 0.5% and 1%) were prepared in this study according to the procedure mentioned in experiment 1. Each xanthan gum (0.25%, 0.5% and 1%) was again mixed with powdered biochar according to the selected seed coating mixture ratio (mixture ratio A) from experiment 1. Further, sucrose has been incorporated into the coating mixture to provide an available energy source for the bacteria.

The following treatments show the further development of the seed coating formulation. Treatments given for the seed samples were; T_1S_0– 0.25% xanthan gum without sucrose, T_2S_0– 0.5% xanthan gum without sucrose, T_3S_0– 1% xanthan gum without sucrose, T_1S_1– 0.25% xanthan gum + 1% sucrose, T_2S_1– 0.5% xanthan gum + 1% sucrose, T_3S_1– 1% xanthan gum + 1% sucrose. Each time the sucrose concentration was maintained at 1% (W/V). Three replicates of 20 surface-sterilized seeds were dipped in the mixture of the above-mentioned coating treatments and air-dried for 24 hours. Obtained coated seeds were examined for their applicability under two criteria; 1) seed surface area covered by the coat, 2) abrasion and dust off and were again checked for germination percentage within 5 days. According to the test results, a better seed coating formula (will be referred to as mixture B) was selected.

2.3 Seed surface area covered by the coat

Seeds after coating application and air drying at 28 – 30°C for 24 hours, the seed surface area coverage by the coating was observed by using 20 seeds from each sample and categorized into a scale of six (100% surface coverage – scale 1, 80% surface coverage – scale 2, 60% surface coverage – scale 3, 40% surface coverage – scale 4, 20% surface coverage – scale 5, 0% surface coverage – scale 6).

2.4 Abrasion and dust off

This test was done to select a suitable seed coating formula which can tolerate mechanical stress during the process and storage. Twenty seeds were surface sterilized, weighed and coated with each coating formula separately, which are mentioned above, and air dried at 28 – 30°C for 24 hours. Then the coated seeds were again weighed and subjected to the abrasion test. To perform the abrasion test, each seed sample was placed in a plastic container and vigorously shaken for 1 minute and again weighed the seeds with the remaining coat. Lost weight was calculated according to the following formula.

Dust off percentage = [(Initial coat weight - Final coat weight)/ Initial coat weight] × 100

2.5 Germination test

Twenty coated and 20 non-coated seeds were selected and placed inside a large Petri plate lined with moistened tissue paper separately and allowed to germinate under sunlight in three replicates. These Petri plates were examined each day for 5 days and the number of germinated seeds were recorded (i.e., 2 mm of the radicle emergence was the criterion for the germination). The germination percentage of each sample was calculated according to the following formula.

Germination percentage = [(No. seeds germinated by final count/ Total No. of seeds) × 100]

2.6 Isolation and identification of Bradyrhizobium japonicum from plant material

Roots with the effective nodules were collected from 35-40 days old soybean plants which were selected randomly from a cultivation in Galenbindunuwewa Sri Lanka (8.28545 N, 80.71498 E) to isolate *Bradyrhizobium japonicum* specifically. The roots of soybean

plants were thoroughly washed with running tap water and from each plant, about 12 active nodules were collected by carefully separating them from the roots. Isolation of *Bradyrhizobium japonicum* was done according to the method described in Somasegaran and Hoben (1994). First, the nodules were surface sterilized by submerging in 95 % ethanol for 10 – 20 seconds. Then the nodules were soaked in 3% (V/V) of NaOCl solution for 3 – 4 minutes to remove possible contaminant microbes. Further, the nodules were rinsed with sterile distilled water in five changes to remove all the chemicals from the nodule surfaces. Subsequently, surface sterilized soybean root nodules were placed in a sterilized Petri dish and crushed by using a surface-sterilized glass rod after adding one milliliter of sterilized distilled water to obtain a watery paste. This watery paste was used to prepare several streak plates by using Yeast Mannitol Agar medium which was modified with Congo Red (CRYMA). CRYMA was considered as a differential medium for rhizobia. In the presence of congo red, *Rhizobium* sp. and *Bradyrhizobium* sp. produced white or light pink colour colonies, while other contaminant species produced red color colonies when incubated under dark conditions. Prepared plates were incubated in dark conditions at room temperature (28 ± 2 ℃). Within 3-6 days from inoculation, colonies of *Rhizobium* sp. were observed and the colonies which were appeared within 4 – 6 days from inoculation were considered as *Bradyrhizobium* spp. as they were comparatively slow-growing bacteria than *Rhizobium* sp. Obtained colonies were re-inoculated several times on the CRYMA medium until a pure culture was obtained. Identification of *Bradyrhizobium japonicum* was done based on morphological, physiological and biochemical characteristics mentioned in Bergey's manual of Systematic Bacteriology (1984).

2.7 Rhizobia inoculation for the selected seed coat formulation (Mixture B)

The microbial culture was prepared by incubating at 30℃ in a shaking water bath (rpm 100) manufactured by Gemmy Industrial Corp. (Model YCW-012S) for 4 days into 50 ml of yeast mannitol broth. The inoculum was aseptically inoculated to the coating mixture according to MacFarland standard solution 1, hence, considered that the mixture contained 3×10^8 CFU ml^{-1}. The prepared seed coating mixture was applied to surface sterilized seeds and air dried for 24 hours.

2.8 Survivability of Bradyrhizobium japonicum on seed coat at 4℃ and 30℃

The coated seeds were initially tested for survivability of bacterium in the coat after 24 hours to check the effect on the bacterial inoculum after the coating process. For that, three replicates were carried out each with ten coated seeds. Then, the seeds were stored at two different temperatures; 4℃ and 30℃. Three replicates of coated seeds each containing 10 seeds were retrieved from the storage conditions on day 03, 05, 10, 15, and 20 and the survivability of the bacterium on the coat was checked by using serial dilution technique. For that, seeds from each sample were placed in a surface sterilized 100 ml beaker separately and shaken vigorously with 10 ml of sterilized distilled water for 10 minutes at 120 rpm on a rotary shaker. The obtained solution was used to prepare the dilution series.

2.9 Germinability of coated seeds stored at 4℃ and 30℃

Coated seeds were stored at 4℃ and 30℃ separately. Three replicates (20 seeds per replicate) were retrieved from the storage conditions on day 15 and a germination test was conducted (2 mm of the radicle emergence was the criterion for the germination). A viability test was conducted for non-germinated seeds.

2.10 Statistical analysis

Statistical analysis was done with one-way ANOVA procedure using MINITAB 18 statistical software. Tukey mean separation procedure, and Mann-Whitney test were conducted to identify the significant differences among treatments appropriately. To analyze the seed surface coverage by the seed coating and to determine the survivability of the inoculum, Kruskal-Walli's test was used. All the data were tested for normality using the Anderson-Darling test before analysis. All the germination data were arcsine transformed prior to data analysis.

3. RESULTS

3.1 Seed coat formulation- Experiment 1

According to the results the seed surface coverage percentages were significantly different among the treatments (DF = 4, h value = 86.61, p value = 0.000). The highest total seed surface coverage was observed in xanthan gum: biochar (80 g:20 g), and lowest was observed in xanthan gum: biochar (20 g:80 g) by considering the applicability (i.e., the total seed surface coverage by the seed coating).

3.2 Seed coat formulation- Experiment 2

According to the results, all the treatments have shown total (100%) coverage of the seed surface. However, by considering the average dust-off percentage of each treatment after being subjected to an abrasion test, there was a significant difference between the treatments (f value = 7.53, p value = 0.002; Figure 01). T_1S_0; the coating material which contained 0.25% of xanthan gum without sucrose showed the highest mean of dust-off percentage which is significantly different from all other treatments. T_1S_1 which contained 0.25% xanthan gum with 1% sucrose showed the second highest average mean of dust-off percentage. The mean of dust-off percentage decreased in the T_2S_0, T_3S_0, T_2S_1 and T_3S_1 treatments, subsequently. However, except T_1S_0, there is no significant difference among other treatments in mean dust-off percentage. Thus, this indicates that, 0.5% and 1% xanthan gum concentrations are more effective than 0.25% xanthan gum concentration in reducing the average dust-off percentage despite the presence of sucrose in the film coat. Although T_3S_1 had the lowest mean of all, the standard deviation was higher than T_2S_1, which has the second lowest mean, hence, T_2S_1 was chosen as the best seed coating material.

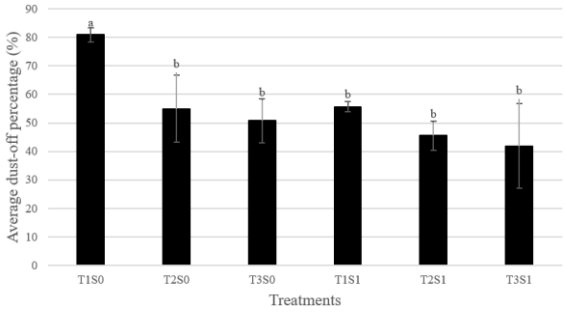

Figure 01. Average dust-off percentage (Different letters indicate significant differences between treatments ($p < 0.05$)) (T1S0 – 0.25% xanthan gum without sucrose, T2S0 – 0.5% xanthan gum without sucrose, T3S0 – 1% xanthan gum without sucrose, T1S1 – 0.25% xanthan gum + 1% sucrose, T2S1 – 0.5% xanthan gum + 1% sucrose, T3S1 – 1% xanthan gum + 1% sucrose). Vertical bars represent the standard deviation of mean values of the treatments.

3.3 Survivability of Bradyrhizobium japonicum in the seed coating under different storage temperatures

According to previously obtained results, T_2S_1 seed coat treatment was chosen, and the effect of different storage temperature conditions was analyzed. The survivability of the inoculum was significantly different with the number of days from coating application in both coated seed samples which were stored under 4°C (DF = 6, h value = 18.15, p value = 0.006) and 30°C (DF = 4, h value = 10.17, p value = 0.038, see Table 01). Although, within the first three days from the coating application, the survival of the inoculum was high on the seed coat (~ 1×10^7 CFU/ml), after day 03, there was a drastic reduction of the inoculum survivability. When considering the storage temperature, there was

no significant difference of the survivability of the bacterium between the treatments up to 10 days. From day 15, the survivability of the bacterium was unable to be tested in the coated seed sample stored under 30℃ because the seed stock was heavily contaminated with fungi (Table 1).

Table 01. Survivability of *Bradyrhizobium japonicum* in the seed coating under 4℃ and 30℃

Number of days from coating application	Mean survivability of *Bradyrhizobium japonicum* at 4℃ [CFU/g (10^7)] ± SD	Mean survivability of *Bradyrhizobium japonicum* at 30℃ [CFU/g (10^7)] ± SD
Day 1	0.913 ± 0.151	0.913 ± 0.151
Day 3	1.08 ± 0.141	0.89 ± 0.220
Day 5	0.105 ± 0.018	0.067 ± 0.005
Day 10	0.062 ± 0.020	0.066 ± 0.020
Day 15	0.073 ± 0.012	0
Day 20	0.07 ± 0.016	0

Figure 2. (a) Coated seeds which allowed to germinate on moistened tissue papers in light/dark (12h/12h) at room temperature (28±2℃); (b), (c) Germinated coated seeds in light/dark (12h/12h) at room temperature (28±2℃)

3.4 Germination percentage after 15 days of coating application of coated and non-coated seeds stored under 4℃ and 30℃

The effect of both seed coating application and the storage temperature for the seed germination was tested on the 15th day after seed coating application (Figure 02). According to the analysis, there was a significant difference between the mean germination percentage of coated and non-coated seeds stored at 4℃ and 30℃ (f value = 7.86, p value = 0.009). The highest mean of germination percentage is shown by the non-coated seeds stored at 4℃ (90%). The mean germination percentages of coated seeds stored at 4℃ and non-coated seeds stored at 30℃ were 80% and 78.3% respectively and the lowest mean of germination percentage was shown by the coated seeds stored at 30℃ (46.67%) (Figure 03).

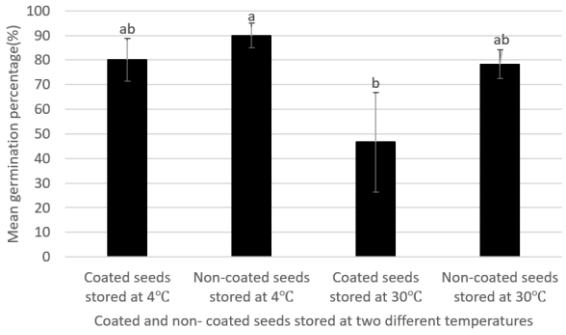

Figure 03. Germination percentage after 15 days of coating application of coated and non-coated seeds stored under 4℃ and 30℃ (Different lowercase letters indicate significant differences between treatments ($p < 0.05$). Whiskers represent the standard deviation of mean values of the treatments.

4. DISCUSSION

The ability of the seed coating mixture to cover the seed surface uniformly with less dust-off effect is essential when considering the inoculation of legume crops with rhizobia via a basic seed coating. Most importantly, the seed coating should not impede the process of seed germination while encasing the inoculum on the seed.

Therefore, the selection of a suitable binder: filler ratio combination is crucial when designing a basic low-cost seed coating formula. According to the results obtained by observing the seed surface coverage by the seed coating with different ratios of binder: filler, only 80 g:20 g (xanthan as binder: biochar as filler) has shown uniformly distributed coating mixture on the seed. Another important aspect is the concentration of the binder which was the xanthan gum in our formulation.

It was revealed that, all the treatments showed total surface coverage of the seed coat, hence, the best coating treatment was selected by analyzing the dust-off percentage of each treatment. Determining the percentage of dust-off is an important aspect, because, if the final product leaves lots of dust behind and not resistant to abrasion, the inoculum on the seed coating would be reduced. Despite the addition of sucrose, the treatments formulated with 0.25% xanthan gum concentration showed the highest percentage of dust-off. All the other treatments except T_1S_0, showed no significant difference among them. The lowest mean dust-off percentage was shown by the 1% xanthan gum + 1% sucrose (T_3S_1) but the standard deviation of the treatment was higher hence, the study continued with treatment T_2S_1 (0.5% xanthan gum + 1% sucrose) which showed the second lowest dust-off percentage. The effect of the coating formula was again checked for germination and according to the results, there was no significant difference among the treatments and the control. Thus, choosing the best coating formula was determined based on the dust-off percentage results. According to a previous study done to explore the activity of different additives for the survival of bacteria in desiccated states, sucrose has been identified as a better suspending solution (Heller 1941). A study done by suspending cells in 10% sucrose solution and in water has revealed that the survivability in sucrose solution was 24 – 44% whereas 0.1% in water (Vincent 1958). However, in another study, different concentrations of sucrose along with *Bradyrhizobium japonicum* were encapsulated in alginate beads (Chhetri *et al*. 2019) and they have tested five different sucrose concentrations (1%, 2%, 3%, 5% and 10%) with the inoculum and have discovered that the inoculum survival with the time is higher in lower sucrose concentrations (190 days) compared to 5% and 10% concentrations (145 days) showing that the inoculum survives well with lower sucrose concentrations while maintaining a preferable moisture content for the bacterium.

The storage temperature of the seeds is another critical aspect which was considered during the study because it affects the viability of the seed as well as the bacteria (Mbofung *et al.* 2013). The influence of the storage temperature for the seed germination and for the survivability of the inoculum was tested and the results indicate that both coated and non-coated seeds stored at 4℃ have higher percentage of seed germination compared to seeds stored at 30℃. According to previous studies, higher storage temperatures influence in reducing seed germinability and viability (Ndimande *et al*. 1981; Patel *et al.* 2017; Delouche 2021). Higher storage temperatures tend to increase the activity of enzymes, metabolic activities and the formation of free radicals (Bernal-Lugo and Leopold 1998). In addition, relative humidity also important in maintaining the seed quality, because elevated levels of relative humidity can cause significant decline in seed quality (Assefa and Srinivasan, 2016; Delouche 2021). Apart from these two factors seed genetics, seeds produced environmental conditions, harvesting time and pathogens also has a strong influence

to the seed viability, vigor and seed longevity (Cho and Scott 2000; Qun *et al*. 2007).

The present study showed that there were no effects of storage temperature on bacterium survivability in seed coats for short-term storage. However, coated seeds stored at higher temperatures can cause a reduction of seed viability due to fungal infections. Previous studies have shown that the inoculum survivability is higher when stored under lower temperatures. Soil-peat cultures inoculated with *Rhizobium meliloti* stored at 2℃ and for 18 months have showed 640×10^7 - 1.6×10^8 viable cells/g while the cultures stored at 10℃ - 28℃ have showed 165×10^6 - 302×10^6 cells/g (van Schreven 1970). Chao and Alexander (1984) tested out the survivability of seed coat-applied soil-based inoculants with several *Rhizobium* spp. stored at 4℃, 25℃, 35℃. The highest inoculum survival count was served from the seeds stored at 4℃ compared to 25℃ and 35℃ (Chao and Alexander 1984). Other than the temperature, several other aspects of biotic and abiotic properties such as desiccation and seed coat exudates influence the survivability of the inoculum on the seed coat (Deaker *et al*. 2004).

Maintaining the survivability of an inoculum of plant-beneficial microorganisms (PBM) is quite a challenging task whether the inoculum is applied directly to the field or delivered via seeds because even a small change in that microenvironment may lead to drastic differences in the inoculum. PBM are introduced to the field via solid or liquid applications, foliar applications or seed applications (Mahmood *et al*. 2016). For a long-lasting inoculum survival on the field until the bacterial population infect legume roots and colonizes, it should be applied along with a carrier material. According to previous studies, a potential carrier material must have a high water-retention capacity, uniformity in physical and chemical properties, non-toxic to the inoculum and have a neutral or adjustable pH (Stephens and Rask 2000). Although broth culture applications allow multiplying the bacterial inoculum within the broth, once applied, due to rapid desiccation the inoculum does not survive longer. Among powdery/dry carriers, peat inoculums are much popular than other carriers as it works well with the inoculum. Less inoculum survivability can be observed in these dry carriers too as desiccation occurs when exposed to the dry environment hence, the efficiency of nodulating the legume roots by the inoculum is less due to high wastage. This is the main reason why the rhizobia inoculants are added and mixed with seeds immediately before sowing. Therefore, seed application of rhizobia inoculants is a better alternative to avoid wastage. To get good contact with the inoculum on the seed coat, an adhesive substance which is called as a binder is used. Even selecting a suitable binder should be done with care because the binder should not interfere with the growth of the inoculum on the seed surface and facilitate its survivability of it until the seeds are released to germinate on the field. The importance of identifying a better seed coating formula comes in handy at this point because a good seed coating can hold the inoculum for a longer period with a longer shelf-life yet, works efficiently when released to the field by forming effective nodulation on legume roots.

The higher moisture content of the seed coat might lead the seed to imbibe and germinate while in the storge and increase fungal attacks. Less moisture tends to desiccate the cells and reduce the viable cell count on the seed coat. The coat application which has been used in the present study also shows similar behaviour. The inoculum value is $\approx 1 \times 10^7$ CFU/ml within the first 03 days but drastically drops after day 03. However, the seed coat application manages to maintain the inoculum value $\approx 0.7 \times 10^6$ CFU/ml which is still a considerable amount in transferring to the field and infecting legume roots. However, the effectiveness of the seed coat should be further checked by conducting field trials.

One of the major problems that appeared during the study was fungal contaminations on the seed.

These contaminations could be seen on both coated and non-coated seeds. Most of the time, the seeds could germinate, although the seed coat's surface is contaminated. By isolating and observing several morphological features of each fungal isolate, common contaminant fungal species were identified as *Penicillium* sp., *Aspergillus* sp. and *Fusarium* sp. According to many researchers, soybean seeds very much tend to contaminate with fungal species due to their elevated levels of oil and protein content (Liu *et al*. 2017). Studies on the mycoflora of soybean seeds have revealed that several fungal species are associated with soybean seeds, including *Alternaria* sp. and *Fusarium* sp., *Colletotrichum* sp., *Aspergillus* sp., *Penicillium* sp., *Macrophomia* sp., *Chaetomium* sp., and *Rhizopus* sp. (Shovan *et al.* 2008).

They could be categorized as field molds and storage molds (Liu *et al*. 2017) and both types of these fungal contaminations affect seed germination, seed viability, seed vigor and other plant performances (McGee *et al.* 1980; Shovan *et al*. 2008; Rao *et al.* 2015). Apart from that, some of these fungal species are responsible for producing mycotoxins which is a great concern in storing seeds for consumption (Ndimande *et al.* 1981; Alemu 2014; Niyibituronsa *et al*. 2018). Delayed harvesting, harvesting during humid environmental conditions and poor storage conditions (e.g., higher moisture content) are some of the major reasons for these contaminations (Niyibituronsa *et al*. 2018; Liu *et al*. 2017). As a solution, seed surface disinfection could be stated as the most immediate solution. Although the seeds were disinfected using a standard procedure (Somasegaran and Hoben 1994) prior to seed coat application during the study, seed-borne pathogens like *Fusarium* sp. were observed in both coated and non-coated seed samples. This indicates that the disinfection procedure was not that efficient to eliminate seed-borne pathogens totally.

Another reason for contaminations on the seed coat may be the sucrose content of the seed coating mixture, as it is a readily available energy source. According to some research, xanthan gum can also be an energy source for fungi and tends to get contaminated even though it performs better than other binders (Jung *et al*. 1982). Therefore, it is important to apply effective strategies to prevent fungal contaminations to improve shelf-life and maintain seed quality. As a solution, fungicides can be incorporated into the coating mixture. Most of the commercially available seeds for agricultural processes are coated with several commercial fungicides. Triazoles, Dithiocarbamates, Strobilurins and Benzimidazoles are commonly using synthetic fungicides in agricultural processes (Hof 2001; Ribas e Ribas *et al.* 2016). But synthetic fungicides may cause several impacts on the ecosystem. The emergence of fungicide-resistant strains is one of the major problems that the world has been facing today Ribas e Ribas *et al*. 2016; Brauer *et al*. 2019). Therefore, moving towards more eco-friendly and sustainable strategy is particularly important. Based on that, natural antifungal agents such *as Eucalyptus globulus*, *Melia azedarach*, *Calotropis procera* etc. (Ahmad *et al.* 2016) and bio-controlling agents e.g., *Trichoderma harzianum*, *Trichoderma atroviride*, *Pseudomonas aeruginosa* (Begum *et al*. 2010; El-Benawy *et al.* 2020) can be incorporated in the seed coat. But, most importantly, before incorporating a synthetic, a natural anti-fungal agent or a bio-controlling agent, the effect of that anti-fungal agent on the survivability of the seed coat incorporated bacterium; *Bradyrhizobium japonicum* should be thoroughly examined.

5. CONCLUSION

In conclusion, this seed coat application can be used to deliver *Bradyrhizobium japonicum* inoculums to the field along with the seeds. The inoculum survivability remains higher during the first 03 days from coating application. Although the inoculum viability declines after day 03, a sufficient amount of the inoculum

remains in the seed coat to colonize soybean roots. By considering the storage temperature, for the germination and inoculum survival, coated seeds stored under 4℃ is more appropriate to maintain the seed quality and to prevent fungal attacks. However, the study should be continued as a field trial to further check the behavior and the effectiveness of the seed coat on plant growth and yield. As an improvement, different natural antifungal agents can be incorporated into the seed coating mixture to prevent fungal contamination.

6. REFERENCES

[1]. Accinelli, C., Abbas, H.K., Little, N.S., Kotowicz, J.K., Mencarelli, M., Shier, W.T., 2016. A liquid bioplastic formulation for film coating of agronomic seeds. Crop Protection 89, 123-128.

[2]. Accinelli, C., Abbas, H.K., Shier, W.T., 2018. A bioplastic-based seed coating improves seedling growth and reduces production of coated seed dust. Journal of Crop Improvement 32(3), 318-330.

[3]. Adhikari, T., Kundu, S., Rao, A.S., 2016. Zinc delivery to plants through seed coating with nano-zinc oxide particles. Journal of Plant Nutrition 39(1), 136-146.

[4]. Ahmad, L., Pathak, N. and Zaidi, R.K., 2016. Antifungal potential of plant extracts against seed-borne fungi isolated from barley seeds (*Hordeum vulgare* L.). Journal of Plant Pathology and Microbiology, 7(5), 5-8.

[5]. Alengebawy, A., Abdelkhalek, S.T., Qureshi, S.R., Wang, M.Q., 2021. Heavy metals and pesticides toxicity in agricultural soil and plants: Ecological risks and human health implications. Toxics 9(3), 42.

[6]. Alemu, K., 2014. Seed -borne fungal pathogen associated with soybean (*Glycine max* L.) and their management in Jimma, Southwestern Ethiopia. Journal of Biology, Agriculture and Healthcare 4(25), 14-19.

[7]. Amirkhani, M., Mayton, H.S., Netravali, A.N., Taylor, A.G., 2019. A seed coating delivery system for bio-based biostimulants to enhance plant growth. Sustainability 11(19), 5304.

[8]. Assefa, F. and Srinivasan, K., 2016. Effect of relative humidity and temperature on shelf life of sorghum, lentil and niger seeds. International Journal of Applied Agricultural Sciences, *2*(6), 83-91.

[9]. Avelar, S.A.G., Sousa, F.V.D., Fiss, G., Baudet, L., and Peske, S.T., 2012. The use of film coating on the performance of treated corn seed. Revista Brasileira de Sementes 34(2), 186-192.

[10]. Begum, M.M., Sariah, M., Puteh, A.B., Abidin, M.Z., Rahman, M.A., Siddiqui, Y., 2010. Field performance of bio-primed seeds to suppress *Colletotrichum truncatum* causing damping-off and seedling stand of soybean. Biological Control 53(1), 18-23.

[11]. Bergey, D.H., Holt, J. G., 1994. Bergey's manual of determinative bacteriology. Lippincott Williams & Wilkins, Philadelphia. pp.1450.

[12]. Bernal-Lugo, I., Leopold, A.C., 1998. The dynamics of seed mortality. Journal of Experimental Botany 49(326), 1455-1461.

[13]. Berninger, T., Mitter, B., Preininger, C. 2016. The smaller, the better? The size effect of alginate beads carrying plant growth-promoting bacteria for seed coating. Journal of Microencapsulation 33(2), 127-136.

[14]. Brauer, V.S., Rezende, C.P., Pessoni, A.M., De Paula, R.G., Rangappa, K.S., Nayaka, S.C., Gupta, V.K. and Almeida, F., 2019 Antifungal agents in agriculture: Friends and foes of public health. Biomolecules 9(10), 521.

[15]. Chandini, R.K., Kumar, R., Om, P., 2019. The impact of chemical fertilizers on our environment and ecosystem. In: Research Trends in Environmental Sciences, 2nd Edition pp.71-86.

[16]. Chandrika, K.P., Prasad, R.D., Godbole, V., 2019. Development of chitosan-PEG blended films using *Trichoderma*: Enhancement of antimicrobial activity and seed quality. International Journal of Biological Macromolecules 126, 282-290.

[17]. Chhetri, T.K., Subedee, B.R., Pant, B., 2019. Isolation, identification and production of encapsulated *Bradyrhizobium japonicum* and

study on their viability. Nepal Journal of Biotechnology 7(1), 39-49.

[18]. Chao, W.L., Alexander, M., 1984. Mineral soils as carriers for Rhizobium inoculants. Applied and Environmental Microbiology 47(1), 94-97.

[19]. Cho, Y., Scott, R.A., 2000. Combining ability of seed vigor and seed yield in soybean. Euphytica 112(2), 145-150.

[20]. Dawar, S., Hayat, S., Anis, M., Zaki, M. J., 2008. Effect of seed coating material in the efficacy of microbial antagonists for the control of root rot fungi on okra and sunflower. Pakistan Journal of Botany 40(3), 1269-1278.

[21]. Deaker, R., Roughley, R.J., Kennedy, I.R., 2004. Legume seed inoculation technology—a review. Soil Biology and Biochemistry 36(8), 1275-1288.

[22]. Delouche, J.C., 2021. Physiological changes during storage that affect soybean seed quality. Seed Technology Papers 123

[23]. El-Benawy, N.M., Abdel-Fattah, G.M., Ghoneem, K.M., Shabana, Y.M., 2020. Antimicrobial activities of *Trichoderma atroviride* against common bean seed-borne *Macrophomina phaseolina* and *Rhizoctonia solani*. Egyptian Journal of Basic and Applied Sciences 7(1), 267-280.

[24]. Farooq, M., Wahid, A., Siddique, K.H., 2012. Micronutrient application through seed treatments: a review. Journal of soil science and plant nutrition 12(1), 125-142.

[25]. Geisseler, D., Scow, K.M., 2014. Long-term effects of mineral fertilizers on soil microorganisms–A review. Soil Biology and Biochemistry 75, 54-63.

[26]. Guo, J.H., Liu, X.J., Zhang, Y., Shen, J.L., Han, W.X., Zhang, W.F., Christie, P., Goulding, K.W.T., Vitousek, P.M., Zhang, F.S., 2010. Significant acidification in major Chinese croplands. Science 327(5968), 1008-1010.

[27]. Halmer, P., 2006, August. Seed technology and seed enhancement. In XXVII International Horticultural Congress-IHC2006: International Symposium on Seed Enhancement and Seedling Production 771 pp. 17-26.

[28]. Hameeda, B., Harini, G., Rupela, O.P., Kumar Rao, J.V.D.K., Reddy, G., 2010. Biological control of chickpea collar rot by co-inoculation of antagonistic bacteria and compatible Rhizobia. Indian Journal of Microbiology 50(4), 419-424.

[29]. Hara, Y., 2013. Improvement of rice seedling establishment in sulfate-applied submerged soil by application of molybdate. Plant Production Science 16(1), 61-68.

[30]. Hartley, E., Gemell, L.G., Herridge, D.F., 2004. Lime pelleting inoculated serradella (*Ornithopus* spp.) increases nodulation and yield. Soil Biology and Biochemistry 36(8), 1289-1294.

[31]. Heller, G., 1941. A quantitative study of environmental factors involved in survival and death of bacteria in the desiccated state. Journal of Bacteriology, 41(2), 109-126.

[32]. Hof, H., 2001. Critical annotations to the use of azole antifungals for plant protection. Antimicrobial Agents and Chemotherapy 45(11), 2987-2990.

[33]. Jambhulkar, P.P., Sharma, P., Yadav, R. 2016. Delivery Systems for Introduction of Microbial Inoculants in the Field. Microbial inoculants in sustainable agricultural productivity pp. 199–218. Springer, New Delhi. doi: 10.1007/978-81-322-2644-4.

[34]. Jayawardhane, S. and Yapa, N., 2018. Potential of microbial solubilization of rock phosphate for use in sustainable agriculture: does biochar application enhance microbial solubilization? Journal of Advances in Microbiology *8*(2), 1-8.

[35]. Jung, G., Mugnier, J., Diem, H.G., Dommergues, Y.R., 1982. Polymer-entrapped Rhizobium as an inoculant for legumes. Plant and Soil 65(2), 219-231.

[36]. Liang, B., Lehmann, J., Solomon, D., Kinyangi, J., Grossman, J., O'Neill, B., Skjemstad, J.O., Thies, J., Luizao, F.J., Petersen, J., Neves, E.G, 2006. Black carbon increases cation exchange capacity in soils. Soil science society of America journal 70(5), 1719-1730.

[37]. Liu, J., Deng, J.C., Yang, C.Q., Huang, N., Chang, X.L., Zhang, J., Yang, F., Liu, W.G., Wang, X.C., Yong, T.W., Du, J.B., Shu, K., Yang, W.Y., 2017. Fungal diversity in field mold-damaged soybean fruits and

pathogenicity identification based on high-throughput rDNA sequencing. Frontiers in Microbiology 8, 779.

[38]. Lopisso, D.T., Kühlmann, V., Siebold, M. 2017. Potential of soil-derived fungal biocontrol agents applied as a soil amendment and a seed coating to control *Verticillium* wilt of sugar beet. Biocontrol Science and Technology 27(9), 1019-1037.

[39]. Mabrouk, Y., Hemissi, I., Salem, I.B., Mejri, S., Saidi, M., Belhadj, O., 2018. Potential of rhizobia in improving nitrogen fixation and yields of legumes. Symbiosis 107(73495).

[40]. Mahmood, A., Turgay, O.C., Farooq, M., Hayat, R., 2016. Seed biopriming with plant growth promoting rhizobacteria: a review. FEMS Microbiology Ecology 92(8), fiw112.

[41]. Mbofung, G.C., Goggi, A.S., Leandro, L.F., Mullen, R.E., 2013. Effects of storage temperature and relative humidity on viability and vigor of treated soybean seeds. Crop Science 53(3), 1086-1095.

[42]. Mia, M.B., Shamsuddin, Z.H., 2010. Rhizobium as a crop enhancer and biofertilizer for increased cereal production. African Journal of Biotechnology 9(37), 6001-6009.

[43]. McGee, D.C., Brandt, C.L., Burris, J.S., 1980. Seed mycoflora of soybeans relative to fungal interactions, seedling emergence, and carry over of pathogens to subsequent crops. Phytopathology 70(7), 615-617.

[44]. Ndimande, B.N., Wien, H.C. and Kueneman, E.A., 1981. Soybean seed deterioration in the tropics. I. The role of physiological factors and fungal pathogens. Field Crops Research, 4,113-121.

[45]. Niyibituronsa, M., Onyango, A.N., Gaidashova, S., Imathiu, S.M., Uwizerwa, M., Wanjuki, I., Nganga, F., Muhutu, J.C., Birungi, J., Ghimire, S., Raes, K., De Boevre, M., De Saeger, S., Harvey, J., 2018. Evaluation of mycotoxin content in soybean (*Glycine max* L.) grown in Rwanda. African Journal of Food, Agriculture, Nutrition and Development 18(3), 13808-13824.

[46]. O'Callaghan, M., 2016. Microbial inoculation of seed for improved crop performance: issues and opportunities. Applied Microbiology and Biotechnology 100(13), 5729-5746.

[47]. Patel, J.B., Sondarva, J., Babariya, C.A., Rathod, R.R., Bhatiya, V.J., 2017. Effect of different storage conditions and seed treatments on seed viability in soybean [*Glycine max* (L.) Merr.]. Journal of Applied and Natural Science 9(1), 245-252.

[48]. Pathma, J., Sakthivel, N., 2012. Microbial diversity of vermicompost bacteria that exhibit useful agricultural traits and waste management potential. SpringerPlus 1(1), 1-19.

[49]. Pedrini, S., Merritt, D.J., Stevens, J., Dixon, K., 2017. Seed coating: science or marketing spin? Trends in Plant Science 22(2), 106-116.

[50]. Peltonen-Sainio, P., Kontturi, M., Peltonen, J., 2006. Phosphorus seed coating enhancement on early growth and yield components in oat. Agronomy Journal 98(1), 206-211.

[51]. Petter, F.A., Madari, B.E., 2012. Biochar: Agronomic and environmental potential in Brazilian savannah soils. Revista Brasileira de Engenharia Agrícola e Ambiental 16, 761-768.

[52]. Poonia, S., 2011. *Rhizobium*: a natural biofertilizer. International Journal of Engineering and Management Research (IJEMR) 1(1), 36-38.

[53]. Qun, S., Wang, J.H., Sun, B.Q., 2007. Advances on seed vigor physiological and genetic mechanisms. Agricultural Sciences in China 6(9), 1060-1066.

[54]. Rao, T.V., Rajeswari, B., Keshavulu, K., Varma, V.S., 2015. Studies on seedborne fungi of soybean. SSRG International Journal of Agriculture & Environmental Science 2(1), 16-24.

[55]. Rashid, M.H., Kamruzzaman, M., Haque, A.N.A., Krehenbrink, M., 2019. Soil Microbes for Sustainable Agriculture. In: Sustainable Management of Soil and Environment, Springer, Singapore, 339-382.

[56]. Rashid, M.I., Mujawar, L.H., Shahzad, T., Almeelbi, T., Ismail, I.M., Oves, M. 2016. Bacteria and fungi can contribute to nutrients bioavailability and aggregate formation in degraded soils. Microbiological Research 183, 26-41.

[57]. Rehman, A.U., Farooq, M., 2013. Boron application through seed coating improves the water relations, panicle fertility, kernel yield, and biofortification of fine grain aromatic rice. Acta physiologiae plantarum 35(2), 411-418.

[58]. Ribas e Ribas, A.D., Spolti, P., Del Ponte, E.M., Donato, K.Z., Schrekker, H., Fuentefria, A.M., 2016. Is the emergence of fungal resistance to medical triazoles related to their use in the agroecosystems? A mini review. Brazilian Journal of Microbiology 47, 793-799.

[59]. Samarakoon, D. and Yapa, N., 2019. Growth, yield and seed nutrient quality of soybean (*Glycine max* L.) as affected by organic, biofertilizer and synthetic fertilizer application. South Asian Journal of Research in Microbiology, *5*(1), 1-6.

[60]. Sarrocco, S., Raeta, R., Vannacci, G., 2004. Seeds encapsulation in calcium alginate pellets. Seed Science and Technology 32(3), 649-661.

[61]. Scott, J.M. 1989. Seed coatings and treatments and their effects on plant establishment. Advances in Agronomy 42, 43-83.

[62]. Shovan, L.R., Bhuiyan, M.K.A., Sultana, N., Begum, J.A., Pervez, Z., 2008. Prevalence of fungi associated with soybean seeds and pathogenicity tests of the major seed-borne pathogens. International Journal of Sustainable Crop Production 3(4)9 24-33.

[63]. Somasegaran, P., Hoben, H. J., 1994. Isolating and purifying genomic DNA of Rhizobia using a large-scale method. In Handbook for Rhizobia Springer, New York, NY. pp. 279-283.

[64]. Stephens, J.H.G., and Rask, H., 2000. Inoculant production and formulation. Field Crops Research 65(2-3), 249-258.

[65]. Ugoji, E.O., Laing, M.D., Hunter, C.H., 2006. An investigation of the shelf-life (storage) of *Bacillus* isolates on seeds. South African Journal of Botany 72(1), 28-33.

[66]. Van Schreven, D.A., 1970. Some factors affecting growth and survival of *Rhizobium* spp. in soil-peat cultures. Plant and Soil, 32(1), 113-130.

[67]. Verma, G., Sharma, R.P., Sharma, S.P., Subehia, S.K., and Shambhavi, S., 2012. Changes in soil fertility status of maize-wheat system due to long-term use of chemical fertilizers and amendments in an alfisol. Plant, Soil and Environment 58(12), 529-533.

[68]. Vincent, J.M., 1958. Survival of root-nodule bacteria. In: Hallsworth, E.G., (Ed.), Nutrition of the Legumes, pp. 108–123.

[69]. Via, V.D., Zanetti, M.E., Blanco, F., 2016. How legumes recognize rhizobia. Plant Signaling and Behavior, 11(2): e1120396.

[70]. Wiatrak, P., 2013. Influence of seed coating with micronutrients on growth and yield of winter wheat in Southeastern Coastal Plains. American Journal of Agricultural and Biological Sciences 8(3), 230.

[71]. Williams, M.I., Dumroese, R.K., Page-Dumroese, D.S., Hardegree, S.P., 2016. Can biochar be used as a seed coating to improve native plant germination and growth in arid conditions? Journal of Arid Environments, 125, 8-15.

[72]. Zahran, H.H., 1999. *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. Microbiology and Molecular Biology Reviews, 63(4) 968-989.

[73]. Zhou, J., Deng, B., Zhang, Y., Cobb, A.B., Zhang, Z., 2017. Molybdate in rhizobial seed-coat formulations improves the production and nodulation of alfalfa. PloS one 12(1), e0170179.