VOLUME 9 NOMOR 2 DESEMBER 2022 **ISSN 2548 – 611X**

JURNAL
BIOTEKNOLOGI & BIOSAINS INDONESIA

Homepage Jurnal: http://ejurnal.bppt.go.id/index.php/JBBI

EFFECTS OF SEED COATING USING PLANT-BENEFICIAL MICROBES ON THE GERMINATION OF *Centrosema pubescens* SEEDS

Pengaruh Pelapisan Benih Menggunakan Mikroba yang Bermanfaat bagi Tanaman terhadap Perkecambahan Benih *Centrosema pubescens*

Indri Handayani[1]*, Farida Rosana Mira[2], Zhafira Amila Haqqa[1], Nia Asiani[1], Abdul Wahid[1], Mahmud Sugianto[2], Bambang Sukmadi[1], Bedah Rupaedah[1]

[1]Research Center for Applied Microbiology, National Research and Innovation Agency, Cibinong Science Center, Soekarno Science and Technology Park, Bogor 16911, Indonesia

[2]Directorate of Laboratory Management, Research Facilities, and Science and Technology Area. Deputy for Infrastructure Research and Innovation, National Research and Innovation Agency, Jakarta 10340, Indonesia

*Email: indri.handayani@brin.go.id

ABSTRACT

Centrosema pubescens *is a legume plant that is commonly used as animal feed, cover crop, and one of the plants used to reclaim critical land. This study was conducted to increase the germination of* C. pubescens *seeds by coating the seeds using a coating material enriched with a consortium of microbes consisting of nitrogen-fixing bacteria, phytohormones-producing bacteria, phosphate-solubilizing bacteria, and arbuscular mycorrhizal fungi. Germination test on control, seed coating, and microbial seed coating was carried out using the top of the sand method for 28 days. Observational data were tested using ANOVA statistics and LSD tests. Results of the research showed the moisture content of the seeds was 12.45%, the purity of the seeds was 95.11% and the weight of 1000 seeds was 23.74 g. The germination test denoted that the number of normal germinated seeds in seed coating and microbial seed coating was significantly different from the control. In addition, dead seeds in microbial seed coating treatment had the lowest value and were significantly different from other treatments. These results indicate that microbial seed coating can increase seed germination and considerably reduce seed death due to seed-borne pathogens.*

Keywords*: arbuscular mycorrhizal fungi, bacteria, Centrosema pubescens, germination, seed coating*

ABSTRAK

Centrosema pubescens adalah tanaman kacang-kacangan yang dimanfaatkan sebagai pakan ternak, penutup tanah dan salah satu tanaman yang digunakan di lahan kritis. Penelitian ini dilakukan untuk meningkatkan perkecambahan benih *C. pubescens* dengan melakukan pelapisan benih yang diperkaya dengan bakteri penambat nitrogen, bakteri penghasil fitohormon, bakteri pelarut fosfat dan cendawan mikoriza arbuskular. Uji Daya berkecambah dilakukan pada benih kontrol, benih berlapis dan benih berlapis mikroba menggunakan metode penanaman benih di atas pasir selama 28 hari. Data pengamatan dianalisis menggunakan ANOVA dan uji lanjut LSD. Hasil penelitian menunjukkan kadar air benih *C. pubescens* 12,45%, kemurnian benih 95,11% dan bobot 1000 benih 23,74 g. Daya berkecambah benih menunjukkan perlakuan benih berlapis mikroba berbeda signifikan dengan kontrol. Persentase benih mati pada perlakuan benih berlapis mikroba memiliki nilai paling kecil dan berbeda nyata terhadap perlakuan lainnya. Hal ini mengindikasikan bahwa pelapisan benih menggunakan mikroba dapat meningkatkan persentase perkecambahan benih dan mengurangi jumlah benih mati akibat patogen bawaan benih.

Kata kunci: bakteri, cendawan mikoriza arbuskular, Centrosema pubescens, perkecambahan, pelapisan benih

Received: *18 November 2022* ***Accepted:*** *24 December 2022* ***Published:*** *29 December 2022*

INTRODUCTION

Centrosema pubescens plant is known as a legume plant which is generally used as animal feed because of its good nutritional content. In addition, this plant also can improve soil quality by increasing soil organic matter, increasing plant nutrition, reducing soil erosion, and helping to maintain soil moisture. Utilization of *C. pubescens* is also carried out by planting these legumes as ground cover plants at mining sites. A study conducted by Sarjono et al. (2019) showed that cover crops, namely *Arachis pintoi, C. pubescens, Pueraria javanica,* and *Calopogonium mucunoides*, could reduce the rate of soil erosion. Research by Tambunan et al. (2017) for post-mining land improvement using *Pueraria phaseloides, C. pubescens* and *C. mucunoides* vegetation cover proved to provide significant carbon stocks in the soil, increase calcium in post-mining land and nitrogen content in the soil.

It is common to plant *C. pubescens* using seeds. Therefore, good quality seeds are needed to support the initial growth of plants. In addition, seed testing is needed to determine the quality of a type or group of seeds. Seed quality is divided into three, physical quality, physiological quality, and genetic quality (Sudrajat et al. 2015). One of the physical quality tests of seeds is seed purity. The seed purity test is aimed to determine the percentage of pure seed from a seed lot so that it can meet the physical quality of the seed. Moisture content is also one of the important parameters that affect seed ability while being stored. Seeds with a high percentage of moisture content will decline in shelf life as it is easy to have a fungal infection. Furthermore, it can affect seed dormancy and germination which are used to define the vital functions of seeds. Drying can be an alternative approach to decrease moisture content, but it can affect seed germination depending on the temperature used (Siddique and Wright 2003).

The process of seed germination must be supported by favorable environmental conditions and eliminating inhibiting factors. Several studies on the germination of *C. pubescens* seeds showed various percentages of germination. *C. pubescens* has the characteristics of slow-growing seeds compared with siratro (*Macroptilium atropurpureum*) and soybean (*Glycine max)*. The small size and surface area of the seeds and the low permeability of the seed coat are factors that affect the seeds of Centro plants to grow because water becomes difficult to enter. The environment for planting seeds must also be considered, notably if the plants are used for the reclamation of critical land. Based on this, it is necessary to increase the germination of *C. pubescens* seeds which can also provide good growth for plants and better soil quality. Seed coating as an alternative technology that is cheap and easy can be a solution to help enhance crop quality, it can also reduce the use of chemical fertilizers (Pedrini et al. 2017, Rocha et al. 2019) so that can be implemented in precision agriculture (Ma et al. 2019). Seed coating is considered as one of the best methods to promote sustainable agriculture. It has beneficial to improve the physical and physiological properties of seeds by increasing growth indices and alleviating abiotic and biotic stresses (Paravar et al. 2022). Furthermore, plant-beneficial microbes (PBM) inoculation can positively affect plant growth and seed germination in maize (Moradtalab et al. 2020). Lal et al. (2022) also demonstrated control and management of potato diseases using beneficial microbes isolated from rhizosphere soil identified as *Pseudomonas* spp which depicted strong inhibition against *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Sclerotium rolfsii* and *Fusarium* spp. The objective of this study is to examine the effect of seed coating using PBM to enhance the germination of *C. pubescens* seeds.

MATERIALS AND METHODS

Location and time

The research was conducted at the research facility of the National Research and Innovation Agency (BRIN) located at Laboratory for Biotechnology, Building 630, BJ Habibie Science and Technology Park, South Tangerang, Banten, Indonesia. The facilities used in this study were Agromicrobiology Laboratory and Greenhouse. This study was conducted from February 2022 to August 2022.

Seed purity

The purity of the seeds was determined by taking random samples by mixing them until they were homogeneous and then the seeds were divided into 8 parts. Each part was taken sufficiently to collect 25 g of seeds. The seeds were then separated from each component in the form of pure seeds, other seeds, and impurities. The test was carried out with three replicates. Each component was weighed and recorded for further calculation of the percentage of components.

Moisture content

Calculation of moisture content was conducted using the whole seeds. This *C. pubescens* seed is a small seed so it does not require grinding or cutting during sample preparation for measuring moisture content. Determination of seed moisture content was done by weighing 5 g of seed material. Then, it was dried in an oven at a temperature of 131 ± 2°C for 4 hours. After that, the seeds were put in a desiccator for 30 minutes. After drying, the seeds were weighed and recorded. Determination of moisture content was carried out with four replications and followed equation 1.

$$Moisture\ content = \frac{m1 - m2}{m1}\ x\ 100\% \quad (1)$$

Where m1 is seed mass before drying, and m2 is seed mass after drying.

Weight of 1000 seeds

The weight of 1000 seeds was determined based on the ISTA (2014) regulations by weighing 100 seeds with eight replications and calculated based on equation 2. The repetition of the calculation was determined by calculating the coefficient of variation (Cv) value according to equations 3 and 4. A Cv value above 4 indicates that the calculation of the weight of 1000 seeds must be repeated (ISTA 2014). The seeds used to calculate the weight of 1000 seeds were pure seeds. Determination of the weight of 1000 seeds was carried out with four replications.

$$Weight\ of\ 1000\ seeds = 10\ x\ \frac{(\sum_{i=1}^{8} x)}{8} \quad (2)$$

where x is the mass of 100 seeds

$$Cv = \frac{S}{X}\ x\ 100 \quad (3)$$

$$S = \sqrt{\frac{n\sum x_i^2 - (\sum x)^2}{N\ (n-1)}} \quad (4)$$

Where Cv is the coefficient of variation, S is the standard deviation, x is the average weight of one hundred seeds, x_i is the weight of 100 seeds per replication, and N is the number of replications.

Microbial Production

The microbes used in this study were collections of bacteria belonging to the Agromicrobiology Laboratory at the Laboratory for Biotechnology. The growth media, starter media, and fermentation media were done by preparing bacterial growth media. Nitrogen-fixing bacteria were cultured using Nitrogen-free Agar dan Nitrogen-free broth media with a composition of 0.1 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 6 g K_2HPO_4, 5 g malic acid, 4 g KH_2PO_4, 0.2 g $CaCl_2$, 0.01 g $FeCl_3$, 0.02 g $Na_2MO_4 \cdot 2H_2O$, 1 g NaOH and 1000 mL distilled water. The medium used for testing Nitrogen-fixing bacteria was nitrogen-free bromthymol blue (NFB). All ingredients were combined and mixed with distilled water and adjusted to pH 6.8, then sterilized at 121ºC for 15 minutes. Pikovskaya Agar and Pikovskaya Broth Media for phosphate-solubilizing Bacteria were prepared by dissolving 10 g of Glucose, 0.1 g of $MgSO_4 \cdot 7H_2O$, 2 g of $Ca_3(PO_4)_3$, 0.2 g of KCl, 0.0025 g of FeSO4, 0.5 g $(NH4)_2SO_4$, 0.0025 MnSO4, 0.5 g Yeast extract, 0.2 g NaCl in 1000 mL of distilled water and adjusted to pH 6.8. Phytohormones-producing bacteria were prepared using King's broth growth medium with a composition of 10 g Glucose, 0.3 g $MgSO_4.7H_2O$, 3 g K_2HPO_4, 2 g tryptophan, 0.5 g yeast extract, 0.5 g Trisodium citrate. All ingredients were put together and mixed with 1000 mL of distilled water. Inoculation of bacteria was performed by taking one colony of bacteria by using ose needle and inserting it into the 100 mL of starter medium, then incubating the starter culture for 24 hours. The starter inoculum was transferred to the fermentation medium. Five up to ten percent of the starter culture was transferred to 1000 mL of fermentation media and the fermented culture was incubated for 48-72 hours. Arbuscular mycorrhizal fungi (AMF) used were *Glomus* sp and *Gigaspora* sp which were

produced using the pot culture method. Spora of AMF was counted by wet sieving. The density of AMF used in seed coating was 250-300 spores per gram of inoculant.

Seed Coating

In this seed coating, there were three treatments, seeds without coating (control), seed coating, and microbial seed coating (Figure 1). Seed coating was performed by adding starch to the prepared seeds and then stirring until it was evenly distributed. The coating materials consisted of compost powder, zeolite, and gypsum were added slowly. The compost was dried and chopped to get fine particles and the coating materials were sieved using an 80 mesh before being used. In the third treatment, microbial seed coating treatment, the microbial addition step was carried out by adding mycorrhiza. Then, the microbial solutions were added using a spray so that the coating material could stick to the seeds. Stages were repeated until the seeds were completely coated. After the seeds were completely coated, sieving was carried out to separate the remaining coating material that does not stick, then the seeds were dried-air at room temperature. After coating, the microbial seed coating was tested for bacterial viability by weighing 10 g of granule compound biofertilizer products and being put into 90 mL sterile water, then shaking for 1 hour. The viability test was performed using the dilution method with a dilution factor of 10^{-1} – 10^{-6}. The dilution factor put to the growth media of each bacterial growth medium (nitrogen-free agar, pikovskaya agar, and nutrient agar) was 10^{-4} – 10^{-6}. The calculation of the number of cells for each bacteria was conducted in 24-120 hours of incubation by counting the bacterial cells that grew on the growth media of each bacteria.

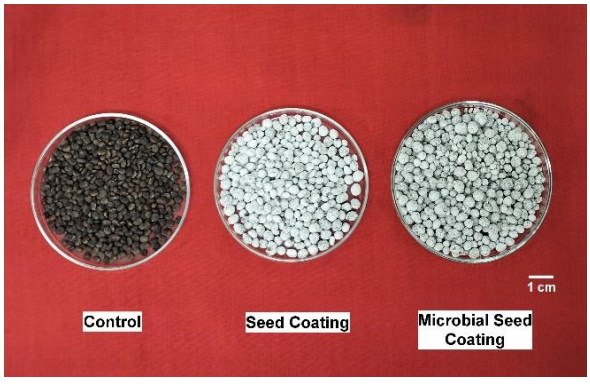

Figure 1. Treatment of *C. pubescens* seeds

Germination Test

The test was carried out by placing 100 seeds in a tray filled with sterile sand with four replicates. The seeds were put on top of the sand. The trays were placed in the screen house and observed daily for 28 days. During observation, the sand media was kept moist. The germination test was carried out by observing normal seedlings, dead seeds, and hard seeds.

Statistical Analysis

Experimental data were displayed as average ± standard deviation (SD) values. The significant differences among the processing means were evaluated with one-way analysis of variance (ANOVA) by multiple sample comparison, and variables of replicates were followed by the least significant difference (LSD) test at $p < 0.05$.

RESULTS AND DISCUSSION

Seed Purity

The data from the calculation of the mass of the purity of *C. pubescens* seeds (Table 1) showed that the sample consisted of 95.11 ± 0.85% pure seeds with other seeds content below 2 ± 0.18% and 3.44± 1.02% of impurities. These other seeds consisted of seeds of other legumes such as seeds of *Acacia sp*. In addition, impurities were dust, husk, and soil (Figure 2).

The purity level of *C. pubescens* seeds in seed samples was quite different from the results of the physical purity evaluation of seeds conducted by Kumar and Sridhar (2015), with an average minimum pure seed fraction of 98% with the remaining 2% of the seed lot being debris, husks, pods, rocks, and soil parts. This phenomenon was due to the different sources of seeds in the market. Seed purity can be improved by removing seed impurities by sorting and separating seeds based on size so that the fraction of seed purity can increase and decrease the fraction of impurities and other seeds.

Table 1. Physical test of *C. pubescens* seed quality (value ± standard deviation)

Parameter	Value
Pure seeds (%)	95.11 ± 0.85
Other seeds (%)	1.45 ± 0.18
Impurity (%)	3.44 ± 1.02
Moisture content (%)	12.45 ± 0.208
Weight of 1000 seeds (g)	23.74 ± 0.076

Figure 2. The results of the separation of the seed components of *C. pubescens*, pure seeds (left), other seeds (middle), impurities (right)

Seed Moisture Content

It was found that the moisture content of *C. pubescens* seeds was 12.45 ± 0.208%. The moisture content of this seed was still in the range of 5 to 13% and belongs to the category of orthodox seeds. According to McCormack (2004), generally for every one percent increase in seed moisture in seeds with moisture content between 5 and 13%, the shelf life of seeds will be reduced by half. Seeds with moisture content above 13% will experience a lower shelf life due to seed-borne fungi and heating due to respiration (Rahmawati and Aqil 2020).

Weight of 1000 Seeds

Results of the research showed that the weight of 1000 seeds was 23.74 ± 0.076 g. Pure seeds generally had various sizes so when weighing, there were some differences for each replication. This also underlies the difference in the value of the physical quality test weighing 1000 seeds with the results of the research of Kumar and Sridhar (2015). In the study of Kumar and Sridhar (2015), the weight of 1000 seeds of *C. pubescens* reached 26.74 g. This difference is common in seeds because the available seed sources are generally different. Lusembo et al. (1995) stated that differences in the size of Centrosema seeds can be resulted from differences in planting systems. The tendency for uniformity of seed size can also be different even in one parent source so that the weight in the same seed production can vary for each test. As for Lusembo's study (1995), the weight of 100 seeds of *C. pubescens* was 2.0 g, 2.6 g, and 2.9 g. Based on this, the weight of 1000 seeds of *C. pubescens* in this study was still in the general range of seed sizes.

PBM on seed coating

The nitrogen-fixing bacteria used as coatings were *Lactobacillus* sp (Figure 3). In soil, nitrogen is one element that is needed for plants to grow and develop. Nitrogen-fixing bacteria are able to bind free nitrogen in the atmosphere and convert it into ammonia (NH_3) which is converted into amino acids and broken down into nitrogen compounds (Tang et al. 2020). *Lactobacillus* sp can enhance growth in corn plants by producing IAA, as a phosphate solubilizing and fixing nitrogen (dos Santos et al. 2020).

Phosphate-solubilizing bacteria (PSB) used in this study were a collection of bacteria belonging to the Agromicrobiology Laboratory. This bacterium belongs to the species *Burkholderia seminalis* (Figure 3). *Burkholderia seminalis* bacteria are phosphate-solubilizing bacteria that are able to dissolve phosphate so that it can be absorbed by plants. The availability of phosphorus is highly dependent on soil pH. In acid soils, phosphate is bound by iron (Fe) and aluminum (Al). PSB is able to release

Figure 3. *Lactobacillus sp* (left), *Burkholderia seminalis* (middle), *Pseudomonas stutzeri* (right)

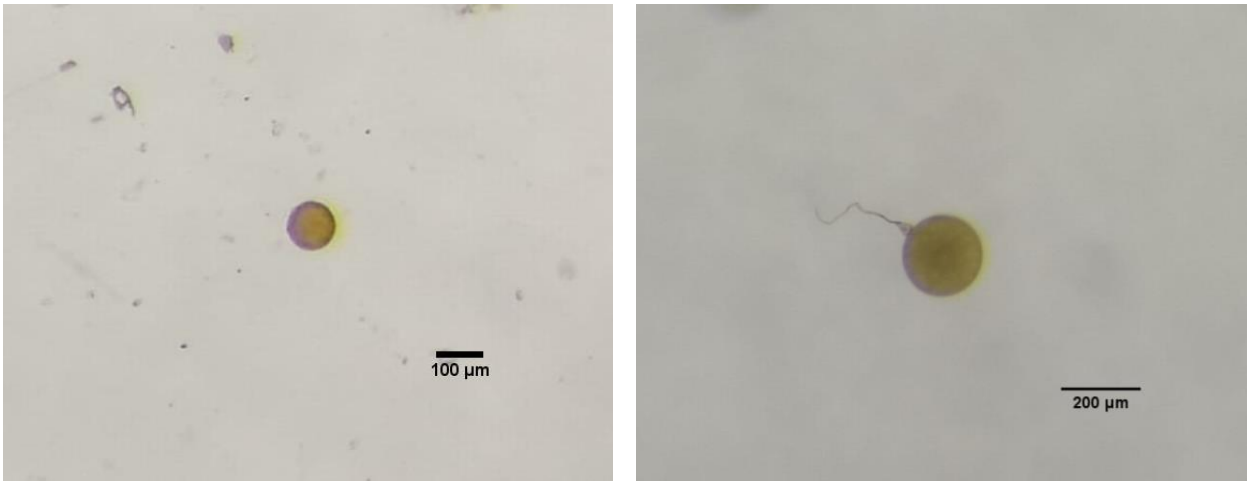

Figure 4. *Glomus* sp (left) and *Gigaspora* sp (right)

many important organic materials that can bind Al, so that it can reduce Al toxicity that occurs in the soil (Tang et. al. 2020).

The phytohormone-producing bacteria used in this study were a collection of bacteria belonging to the Agromicrobiology Laboratory and identified as *Pseudomonas stutzeri* (Figure 3). Phytohormone-producing bacteria have an important role for plants because they can improve soil quality, adaptability (Egamberdieva et al. 2017) and increase plant productivity (Susilowati et al. 2018).

Arbuscular mycorrhizal fungi used were *Gigaspora* sp and *Glomus* sp (Figure 4) species which are the collections of the Agromicrobiology Laboratory. The use of arbuscular mycorrhizal fungi provides positive impact on plant growth and nutrient uptake. Arbuscular mycorrhizal fungi enhanced plant growth, yield, and abiotic stress tolerance by increasing nutrient uptake (Zhang et al. 2020).

All of the bacteria used in the coating process were counted both before and after coating (Table 2). There was a reduced number of bacteria in the microbial seed counting. This might be caused by the microbial inoculum used in the granulation process did not stick perfectly to the coating material because it sticked to the granulator wall. In addition, the existence of a drying process also had an influence on microbial viability after being coated. Moreover, stirring and storage affected the amount of inoculant before and after being coated. According to Ma (2019), survival of microorganisms is affected by several factors such as coating type, coating carrier, drying process, storage condition temperature including humidity, temperature and contaminants.

Germination of *C. pubescens* Seeds

Observation of the germination of *C. pubescens* seeds on day 4 showed that the germination ability of *C. pubescens* seeds in the microbial seed coating treatment was faster and higher than in the seed coating and control treatments. On the 4th day the number of seeds that germinated in control, seed coating, and microbial seed coating was 12%, 23%, and 30.5%, respectively. This phenomenon denoted a higher germination rate compared to Rusdy's (2015) study which only achieved 11% germination on the 10th day. Beneficial microbes play a role in the enhancement of the performances of plants through direct or indirect mechanisms such as dissolving phosphate and producing hormone IAA (Di Benedetto et al. 2017). Invasion of fungal mycorrhiza can promote seed germination and seedling growth via producing plant hormones such as auxins (IAA); gibberellins (GA); Abscisic acid (ABA) (Liu et al. 2022).

Table 2. Total of bacteria before and after coating treatment

Bacteria	Before Coating (CFU/mL)	After Coating (CFU/mL)
Nitrogen-fixing bacteria	3.26×10^9	3.3×10^6
Phosphate-solubilizing bacteria	3.1×10^8	4.4×10^6
Phytohormones-producing bacteria	8.03×10^9	6.8×10^7

The germination test was conducted by observing total hard seeds, normal seedlings, and dead seeds (Figure 5). The highest percentage of hard seeds in the three treatments was in the microbial seed coating treatment, followed by control and seed coating (Figure 6). This was influenced by microbial colonization of plants and seeds. Seeds with coarser skin types will be more difficult to colonize by microbes than seeds with smoother skins (Mancini and Romanazzi 2014). This affected the germination process which caused a delay or decrease in the emergence of sprouts. In the field, these results are related to competitiveness between *C. pubescens* and weeds or unwanted species. Therefore, hard seeds on the availability of *C. pubescens* seeds should be reduced (Rusdy 2015). However, the LSD test indicated that the three percentages were not significantly different.

On the other side, in the number of normal seedlings, it was known that there was a significant difference between control with seed coating and microbial seed coating. The percentage of normal seedlings in control, seed coating, and microbial seed coating was 41.75 ± 2.17%, 52.50 ± 5.59%, and 51.75 ± 5.54%, respectively (Figure 6). Nevertheless, the seed coating and microbial seed coating treatments were not significantly different even though the percentage of seed coating was slightly higher. This was influenced by the content of coating carrier materials such as sand and zeolite which have a role in storing water. Carrier materials such as zeolite can improve germination due to their high water retention properties (Yilmaz et al. 2014). The use of carrier materials such as compost affects the water-holding capacity thereby increasing germination and survival in hard environments (Paradelo et

Figure 5. Hard seeds (left), normal seedlings (middle), and dead seeds (right)

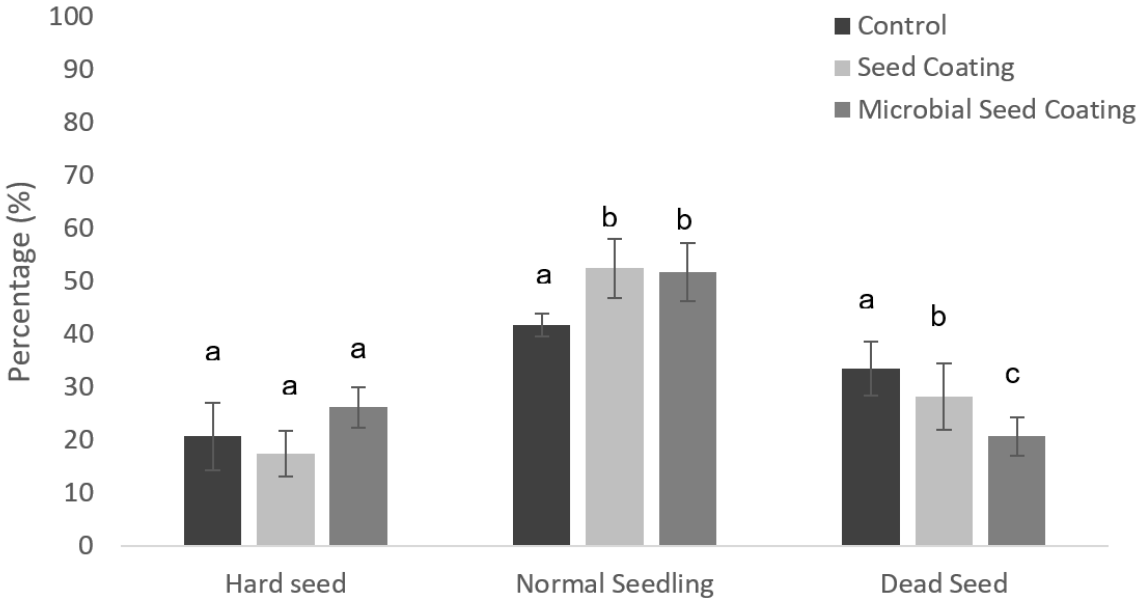

Figure 6. Effect of treatment on germination test of *C. pubescens* seeds over 28 days. Error bars represent standard deviation of mean. Within each graph, differences between mean were resolved using ANOVA followed by LSD test; bars with different lowercase letters are significantly different at $P < 0.05$

al. 2019). The application of *Pseudomonas stutzeri* bacteria to soybean seeds can result in higher seed germination, plant growth and adaptation to high salinity conditions (Lami et al. 2020). Furthermore, mycorrhizal fungi treatments had highly notable interaction effects of *Encyclia phoenicea* germination (Alghamdi 2019).

Microbial seed coating had a major effect in reducing the percentage of dead seeds. This treatment had a lower percentage of dead seeds by 12.75% as compared with the control (33.5 ± 5.12%) while seed coating differed by only 5.25%. In addition, microbial seed coating was significantly different from the other treatments. Dead seeds were mostly caused by seed-borne pathogens which usually show no signs of growth. Seed-borne pathogens associated with external or internal seeds can cause seed abortion, seed rot, seed necrosis, reduction or loss of germination, as well as seedling damage resulting in disease development at later stages of plant growth by systemic or local infections (Amza 2018). Beneficial microbe has the ability to produce antimicrobial metabolites against pathogens (Di Benedetto et al. 2017). Generally, *Pseudomonas* spp naturally can effectively suppress soil-borne pathogens. Some of them have antagonistic properties against soil-borne and seed-borne (Weller 2007, Lal et al. 2022). Moreover, *Burkholderia seminalis* used in microbial seed coating can potentially control the phytopathogenic bacteria in orchid (Araújo et al. 2016). In addition, the use of plant growth-promoting bacteria can increase crop production optimally and the ability of plants to deal with the stress of salinity, drought, and nutritional imbalances (Etesami and Maheshwari 2018).

CONCLUSION

Seed coating and microbial seed coating depicted improvement in *C. pubescens* seed germination at 52.50 ± 5.59% and 51.75 ± 5.54% seed germination, respectively. In addition, microbial seed coating reduced the percentage of dead seeds by 12.75% as compared with the control. Seed coating added with phosphate solubilizing bacteria, nitrogen-fixing bacteria, phytohormone-producing bacteria, and arbuscular mycorrhizal fungi can increase germination and considerably reduce seed death due to seed-borne pathogens.

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