STUDY OF PHYSICO-CHEMICAL FACTORS INFLUENCING THE SEED BIOPRIMING AND ITS IMPACT ON THE SEED GERMINATION AND GROWTH OF *Brassica oleracea* var. kale

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Abstract *Brassica oleracea* var. kale is a green leafy vegetable used in various dishes, salads, and sandwiches due to its high nutritive values. The current study aimed to assess physico-chemical parameters affecting seed biopriming and to study biopriming effect on the growth via evaluating various growth parameters such as germination percentage (GP), average shoot length and average root length of seedlings (ASL and ARL), seed vigour index (SVI), seed metabolic efficiency (SME), relative water content (RWC) at the seedling stage, and further observation of biomass, number of leaves, stem and root length at certain developmental stage. Among the optimized physico-chemical parameters, *Azotobacter* spp. at a concentration 10^8 CFU/mL suspended in 1% NaCl solution of pH 7 were used for inoculation of kale seeds up to 16 hours at 20°C to obtained the maximum growth. Following these optimized conditions, seed biopriming was performed using *Azotobacter* spp, *Rhizobium* spp. and a combined application of both the microbes. The results showed that the biopriming of kale seeds with combined application of *Azotobacter* spp. and *Rhizobium* spp. led to the highest growth parameters followed by the individual application of *Azotobacter* spp or *Rhizobium* spp. compared to untreated control seeds. Overall, biopriming with combination of *Azotobacter* spp. and *Rhizobium* spp. is a viable strategy for improving crop productivity and resilience, contributing to sustainable agriculture and global food security.

Keywords: *Azotobacter* spp.; physico-chemical parameters; biopriming; kale; *Rhizobium* spp.

INTRODUCTION

Currently, the global population has been surging continuously and projected to reach approximately 9.7 billion in the upcoming year 2050 [46]. India, the most populous nation of the world, which estimated to have a population of approximately 1.65 billion in 2050 and it has estimated that this rising population requires nearly 400 million tonnes of food grains production along with approximately 650 million tonnes production of fruit and vegetables in 2050 [19]. So, the need of the hour is sustainable higher production of food that is highly nutritious and easily available to fulfil the daily requirement of everyone. In recent years, significantly higher production of cereals, leguminous and non-leguminous crops is observed globally and similar with fruits and vegetable crops due to various reasons such as improved varieties and production technologies. In this context, we are focusing on green leafy vegetables. In India, approximately 723 species of green leafy vegetables were grown and consumed in different regions [47]. Leafy vegetables are low cost and easy to afford and could be grown outdoors (in the field) and indoors (pots) with smaller space requirements. Mostly, the leafy vegetables distributed all over the world belong to the Brassicaceae family [31]. Brassicaceae family include a wide range of vegetables such as arugula, bittercress, brussels sprouts, cabbage, canola, cauliflower, charlock, collard green, horseradish, kale, kohlrabi, mustard, napa cabbage, rutabaga, shepherd's purse, turnip, wasabi, woad, boy choy, and Chinese cabbage [1]. Most of these species provide edible roots, leaves, stems, buds, flowers and seeds which can be used for different purposes such as oil production, condiments, vegetable and bio-fuel production etc. [29].

Kale belong to brassica family and it is cultivated for their dark green leaves which is the powerhouse/ rich source of dietary fibre, carbohydrates, vitamins (vitamin A, vitamin C, several vitamins of the B complex, vitamin E, vitamin U or S-methylmethionine), flavonoids (quercetin, kaempferol), phenolic compounds, tannins, phytate and nitrogen compounds [7, 24, 38, 52]. Its leafy part could be eaten in different ways such as raw, cooked (boiled or steamed), chips etc. and also used as hot infusion (tea), powder (added to culinary dishes), poultice, capsule and tincture, for various health benefits [54].

The kale is included in the broad leafy vegetable category and its popularity in consumption has grown up with 40% of the leafy green vegetables market [13]. Kale is under limited cultivation because of various growth restraining factors that are associated with soil composition, pH, temperature, and water along with various soil borne diseases that occur from the seed to plant level and thus decrease the germination and yield of the crop.

To overcome the constraints in growth of the plants, at present seeds are treated with fungicides and pesticides as well as heavily supplied with synthetic fertilizers or other chemicals that adversely affect the human health. As an alternate recently, bio-priming which is a safer and economical approach gain much importance in crop enhancement for its role in activation of physiological processes without the emergence of the radicle of seed and used as uniform seed germination with better stand establishment [17, 32]. It has reported that bio-priming enhances the germination percentage, seedling length, seedling vigour index, dry matter production, seed metabolic efficiency, fresh weight and dry weight, micronutrient uptake, grain yield, chlorophyll content, lignin deposition, antioxidant activity, other morphological

characteristics, and moreover stand up against abiotic stress and decrease the disease severity caused by various pathogens [14, 27]. Bio-priming approach has been explored in diverse crops such as finger millet [42], maize [4], beans [33], sorghum [28], tomato [8] and okra [44] so on. Biopriming has also demonstrated significant benefit in Brassicaceae family crops such as cabbage, red cabbage, canola, lettuce, mustard, radish, and rapeseed etc [51, 58, 60, 61]. In kale, till to date there is only one report of exploration of bio-priming [36]. But it has been restricted to seed germination and seedling emergence study only and not much focused on the factors affecting biopriming, growth parameters and other agronomic characteristics. So, keeping these things in mind in the present report physico-chemical parameters were standardized for seed biopriming and studied the impact of biopriming at the seedling and plant level.

MATERIALS AND METHODS

Seeds of *Brassica oleracea* variety kale was procured from the local market of Kurukshetra. Azoteeka™ (*Azotobacter* spp.) and Rhizoteeka™ (*Rhizobium* spp.) were procured from the Centre for Bio-Fertilizer production and technology, Department of Microbiology, Chaudhary Charan Singh Haryana Agriculture University, Hisar, India. The cultures of *Azotobacter* spp. and *Rhizobium* spp. were maintained on the Burk's media and Nutrient Agar, respectively, at 37° C, and stored in a refrigerator at 8 - 10° C.

Seed sterilization and preparation of bacterial cell suspension cultures

Initially, healthy seeds were segregated and disinfected with 70% ethyl alcohol for 1-2 minutes, followed by 0.2% mercuric chloride (4-5 minutes). The seeds were washed four to five times with autoclaved distilled water for 30-60 seconds each time in aseptic conditions under a laminar air flow cabinet, following the procedure of Mehta *et al.* (2022) [30]. A total of sixty sterilized seeds in three replicates (20 seeds per replicate) were taken for each bacterial treatment.

To prepare the bacterial cell suspension cultures, *Azotobacter* spp. and *Rhizobium* spp. were grown individually in the flask containing 50 mL of Burk's media and Nutrient Broth respectively, incubated the cultures at 37±2 °C with continuous shaking overnight using orbital incubator shaker. The bacterial pellets were harvested by centrifugation at 5000 rpm for 10 min at 4°C and adjusted the concentration 1×10^6, 1×10^7, 1×10^8, 1×10^9 and 1×10^{10} CFU/mL via resuspension of pelleted bacteria using the fresh liquid Burk's media and measurement of OD at 600nm.

Standardization of physico-chemical parameters affecting seed biopriming

Various physico-chemical parameters affecting seed biopriming were standardized using *Azotobacter* cultures. The sterilized seeds were treated with different concentrations (1×10^6, 1×10^7, 1×10^8, 1×10^9 and 1×10^{10} CFU/mL) of *Azotobacter* spp. and kept in incubator shaker at 150 rpm for a range of time periods (4, 8, 12, 16, 20 and 24 hours) and temperature range of (15°C, 20°C, 25°C and 30°C). Among the other variable parameters, various priming solutions (Burk's media or 1% NaCl saline or Hoagland's solution) used for bacterial cells suspension preparation and a range of pH levels (5.5, 6, 6.5, 7, 7.5, 8, and 8.5) was also tested. After incubation of seeds with bacterial suspension for each treatment, seeds were dried on a blotting paper and sown in a potting mix consisting of sand, cocopeat, and vermicompost in 1:2:1 ratio. After 10^{th} day of seed sowing, recorded the data and concluded at which parameters highest seed germination and seedling growth occurs, which was used for further analysis.

After standardization of the parameters with *Azotobacter* spp. the optimized protocol was also applied to *Rhizobium* spp. either alone or in combination with *Azotobacter* spp. In both the cases of bacterial treatment, on the 10^{th} day of seed sowing, different parameters such as germination percentage (GP), average shoot and root length (ASL and ARL) of seedlings, seed vigour index (SVI), seed metabolic efficiency (SME), and relative water content (RWC) were analyzed. Around the 40^{th} day of seed sowing, parameters such as biomass, number of leaves, shoot and root length were observed to evaluate the effect of the biopriming.

Evaluation methods

Germination percentage (GP) – The number of germinated seeds (seedlings) were counted from the total number of seeds used for germination in each experiment. Germination percentage was calculated as follows and determined the average value of GP from the data of experiments done in triplicate:

$$GP = (\text{Total number of seedlings/total number of seeds}) \times 100.$$

Average shoot and root length of seedling (ASL and ARL) – After seed germination, the shoot and root length of all the seedlings was measured via scale in centimeter (cm) for each treatment. The average values of the data were used for further analysis of seedling vigour index and seed stamina index.

Seed vigour index (SVI) – Seed vigour index also called seed viability or power of seed for germination. It is the ability of seedling emergence from the soil. It was calculated as follows:

$$\text{Seed vigour index} = [\text{Average shoot length of seedlings (ASL)} + \text{Average root length of seedlings (ARL)}] \times \text{GP}.$$

Relative water content (RWC) – The fresh weight (FW) of the seedlings of each treatment was measured using the weighing balance. After measuring their fresh weight (FW), the seedlings were kept in water for 4 hours and measured their turgid weight (TW). This was followed by drying in a hot air oven at 55 °C until a constant dry weight (DW) was achieved [15]. It was calculated as follows:

$$RWC = \{[\text{Fresh weight (FW)-Dry weight (DW)}] / [\text{Turgid weight (TW)-Dry weight (DW)}]\} \times 100$$

Seed metabolic efficiency (SME) – It is the 1 unit (g) of dry seed weight that is required for producing one gram of dry root and shoot is known as metabolic efficiency of the seed. It was calculated by following [57] as:

SME = Seedling dry weight (g)/ Seed material respired (SMR)

Seed material respired (SMR) = Seed dry weight before germination (SDW) - {Seedling dry weight + Remaining seed dry weight (RSW)}

Biomass – Approximately after 40th day of seed sowing, ten plants were selected and gently uprooted from each treatment and washed the roots to remove any dirt or soil and then dried them in hot air oven at 55°C until a constant biomass was obtained. Next, calculated the mean of the values for further analysis.

Shoot and root length of mature plant – Ten adult plants were selected from each treatment and their average shoot and root length were measured in centimeter (cm) via a measuring scale.

Number of leaves – Ten mature plants were chosen from each treatment, and their leaves were counted for the evaluation of their overall growth and biomass.

Data analysis

The experiments were conducted in three independent replicates and post-hoc analysis was performed using Duncan's Multiples Range Test (DMRT) to identify homogenous subsets among the treatment groups. The analysis was performed using IBM SPSS Statistics 27.0.1 at a significant level of $P \leq 0.05$ and expressed as means ± standard deviation (SD) [50].

RESULTS

In the present study, to ensure the efficient biopriming of *Brassica oleracea* var. kale seeds, various physico-chemical parameters related to seed germination were assessed using *Azotobacter* spp. as depicted in Figure 1. *Azotobacter* spp. plays a key role in enhancing soil fertility by fixing nitrogen and making nutrients more available to plants. The various physico-chemical parameters optimized were as under:

Effect of microbial concentrations on seed biopriming

A range of bacterial cells concentrations from 10^6 to 10^{10} CFU/mL were tested. In this study, maximum value of GP 75.22±0.79% was observed at the concentrations of 10^9 CFU/mL with non-significant difference in GP obtained using 10^8 CFU/mL (74.67±2.36%), followed by 10^{10} (66.89±0.16%), 10^7 (62.22±2.08%), and 10^6 (43.89±0.79%) (Fig. 1A). The maximum value of ASL (6.40±0.48 cm) and ARL (5.1±0.08 cm) were achieved at 10^8 CFU/mL, followed by 10^9, 10^7, 10^{10} and 10^6 CFU/mL with values of ASL (5.87±0.52 cm, 5.42±0.43 cm, 4.53±0.29 cm and 3.52±0.19 cm) and ARL (4.86±0.04 cm, 4.33±0.21 cm, 3.99±0.08 cm and 2.54±0.29 cm), respectively (Fig. 1B). These findings suggests that lower concentrations (10^6 to 10^7 CFU/mL) of microbes exhibits lower GP and seedling growth, while increase in concentrations (10^8 and 10^9 CFU/mL) increases the GP but a decline of seedling length was observed at 10^9 CFU/mL, further increases in concentration (10^{10} CFU/mL) decreased the both GP and seedling growth. Thus, 10^8 CFU/mL concentration was considered as the optimal concentrations at which both GP and seedling growth were maximum.

Effect of temperature on seed biopriming

Among the varying temperature range, *Azotobacter* strainat optimum concentration (10^8 CFU/mL) showed the highest GP (91.67±1.36%) at 20°C. With the increase in temperature from 25°C to 30°C significantly lower value of GP 82.22±1.57% and 64.44±0.79%, respectively was observed (Fig. 1C). However, as the temperature increased from 25 to 30 °C the values of ASL (5.87±0.52 cm and 3.6±0.28 cm) and ARL (4.77±0.12 cm and 3.42±1.03 cm) with respect to both the temperatures were reduced significantly compared to the ASL (7.57±0.12 cm) and ARL (6.27±0.05 cm) obtained at 20°C (Fig. 1D). At 15°C, significantly lower value of GP (85±2.36%), ASL (6.03±0.52 cm) and ARL (5.31±0.34 cm) compare to temperature at 20°C.

Effect of seed biopriming duration

The seeds were bioprimed with *Azotobacter* spp. (10^8 CFU/mL concentration) at 20°C for various durations ranging from 4 to 24 hours. The incubation of seeds up to 16 hours had resulted maximum effect of biopriming with highest GP (95.56±0.79%), ASL (9.0±0.08 cm), and ARL (7.97±0.17 cm) (Fig. 1E and 1F). There was no significant difference found in GP between 16 and 20 hours of incubation but decrease in seedling growth was observed at 20 hours, further increasing the duration of incubation rather increasing, decreased the overall biopriming effect.

Effect of priming solution and their pH on seed biopriming

Among the effect of priming solutions (Burk's media, 1% NaCl saline and Hoagland's solution) and their pH on seed biopriming (Fig. 1). The best response on GP, ASL and ARL (96.67±2.59%, 14.33±0.29 cm, 11.13±0.05 cm, respectively) was observed when the cells of *Azotobacter* spp. were suspended in 1% NaCl saline solution at pH 7, followed by the Burk's media (74.44±1.29%, 13.07±0.05 cm, 10.47±0.21 cm), and Hoagland's solution (66.67±1.7%, 12.83±0.25 cm, 9.6±0.28 cm) (Fig. 1G and 1H). Among the effect of pH of the priming solutions, an increase in the pH from 5.5 to 7 had increased the germination percentage and seedling but further increase in pH of the solutions from 7 to 8.5 rather enhancing declined the germination and seedling growth (Fig. 1G and 1H).

Effect of bacterial strain used for biopriming

The above optimized protocol of seed biopriming using *Azotobacter* spp. was applied to the *Rhizobium* spp. either alone or in combination with *Azotobacter* spp. at a concentration of 10^8 CFU/mL suspended in a 1% saline solution having pH 7 and incubated for 16 hours at 20°C. The findings of this study suggested that

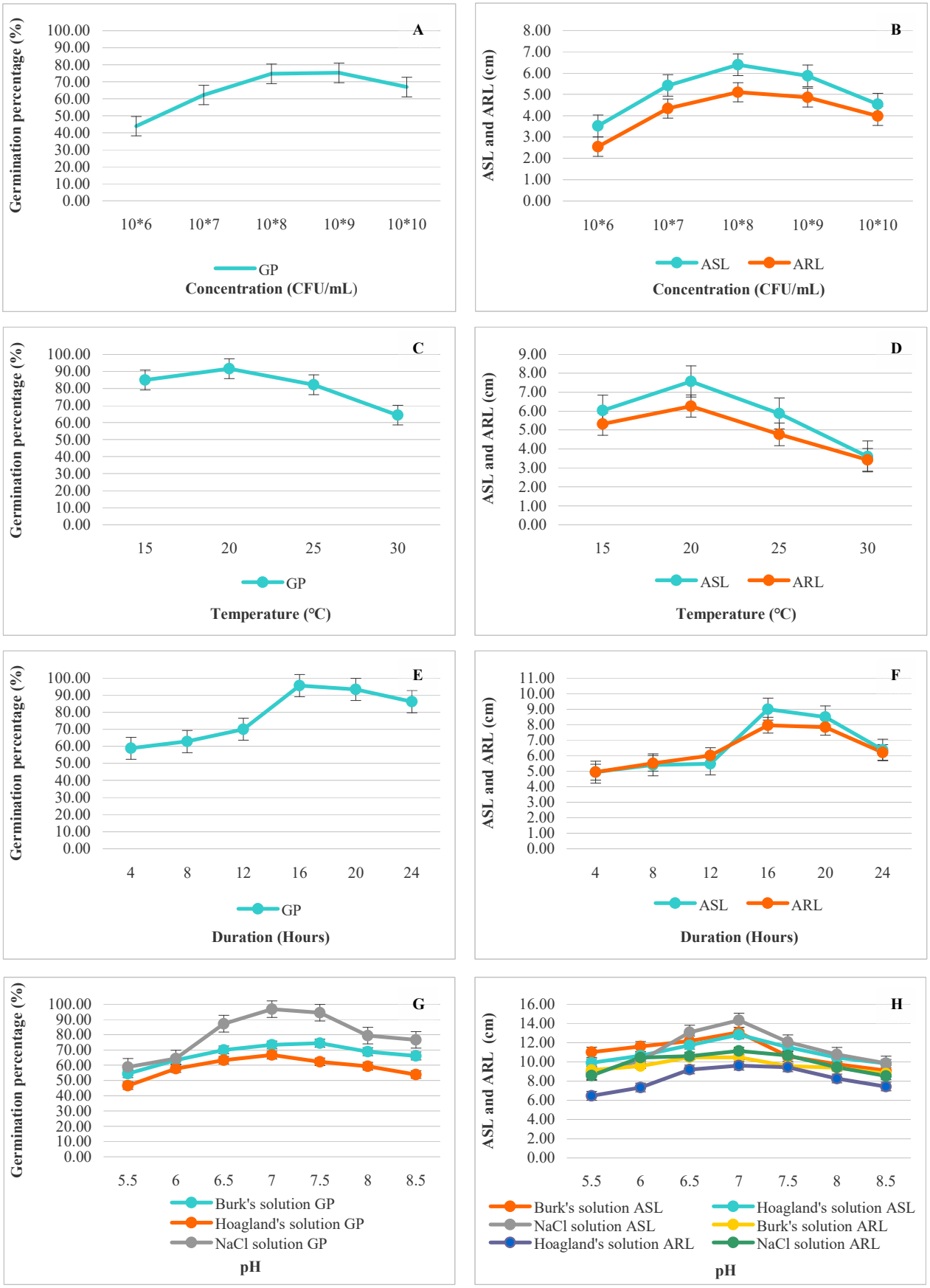

Figure 1. Optimization of various parameters affecting GP, ASL and ARL in seed biopriming of *Brassica oleracea* (kale) using *Azotobacter* species.

seed biopriming with the combined application of *Azotobacter* spp. and *Rhizobium* spp. has resulted highest GP value of 98.89±0.96%, followed by individual application of *Azotobacter* spp. (96.77±2.59%) or *Rhizobium* spp. (73.33±1.41%) compared to control (35±1.36%) (Table 1).

The seed viability or seed vigor index (SVI) depends on GP, seedling length (SL) i.e., higher the GP and SL than higher the SVI and vice versa. In the present study, biopriming with mixture of *Azotobacter* spp. and *Rhizobium* spp. has resulted in maximum ASL of the seedlings (15.3±0.49 cm), followed by the individual application of *Azotobacter* spp. and *Rhizobium* spp. in comparison to untreated (3.73±0.69 cm) (Table 1 and Fig. 2). Similarly, the combined application of *Azotobacter* and *Rhizobium* also

Table 1. Effect of seed biopriming using different bacterial strain on germination parameters of *Brassica oleracea* var. kale. Data based on three replicates of 20 seeds in each and taken at the end of 10th day of seed sowing*.

Treatment	GP (%)	ASL (cm)	ARL (cm)	SVI	RWC (%)	SME (g/g)
Control	35 ± 1.36^{a}	3.73 ± 0.69^{a}	3.13 ± 0.4^{a}	244.44±29.74^{a}	74.35±0.13^{a}	0.5±0.21^{a}
***Azotobacter* spp.**	96.77±2.59^{c}	14.33± 0.29^{c}	11.13±0.05^{c}	2463.85±53.73^{c}	85.59±0.43^{b}	2.5±0.04^{c}
***Rhizobium* spp.**	73.33±1.41^{b}	11.27±0.19^{b}	9.73 ± 0.41^{b}	1540.4± 55.72^{b}	83.47±0.76^{b}	1.17±0.12^{b}
***Azotobacter* spp. + *Rhizobium* spp.**	98.89±0.96^{c}	15.3 ± 0.49^{c}	12.2± 0.73^{c}	2718.44± 95.8^{d}	86.06±0.29^{b}	3.33±0.07^{d}

*Data are presented as mean ± SD. The mean comparisons were conducted using DMRT and values within the same column with different alphabetical superscripts are significantly different at P < 0.05. GP = germination percentage, ASL = average shoot length, ARL = average root length, SVI = seed vigour index, RWC = relative water content, and SME = seed metabolic efficiency.

Figure 2. Effect of biopriming on average shoot length and root length of seedling in *Brassica oleracea* at the end of 10th day with various treatments: control (A), *Azotobacter* spp. (B), *Rhizobium* spp. (C), Combination of *Azotobacter* spp. and *Rhizobium* spp.(D)

Figure 3. Effect of biopriming in *Brassica oleracea* at mature plant stage (at the of 40th day) with various treatments: control (A), *Azotobacter* spp. (B), *Rhizobium* spp. (C), Combination of *Azotobacter* spp. and *Rhizobium* spp.(D)

Table 2. Effect of seed biopriming using different bacterial strain on the growth of *Brassica oleracea* var. kale. Data were taken at the end of 40th day of seed sowing*

Treatment	Biomass (g)	Shoot length (cm)	Root length (cm)	Average number of leaves/plants
Control	19.25±1.01^{a}	14.6±0.32^{a}	8.3±0.87^{a}	5
***Azotobacter* spp.**	75.56±1.76^{c}	43.3±0.61^{c}	18.6±0.22^{c}	10
***Rhizobium* spp.**	47.77±2.65^{b}	24.5±0.54^{b}	14.3±0.45^{b}	7
***Azotobacter* spp. + *Rhizobium* spp.**	137.32±2.03^{d}	58.8±0.12^{d}	19.3±0.19^{c}	11

*Experiments were conducted using three independent replicates. Data are presented as mean ± SD. Mean comparisons were conducted using DMRT and values within the same column with different alphabetical superscripts are significantly different at P < 0.05.

possesses the maximum ARL of seedlings i.e., 12.2±0.73 cm followed by individual application of *Azotobacter* (11.13±0.05 cm) or *Rhizobium* (9.73±0.41 cm) compared to control (3.13±0.4 cm).

The combined application of *Azotobacter* spp. and *Rhizobium* spp. possessed the maximum value of GP, and SL which correspondingly resulted in highest value of SVI (2718.44±95.8) followed by the individual application of *Azotobacter* spp. (2463.85±53.73) and *Rhizobium* spp. (1540.4±55.72) than control SVI (244.44±29.74) (Table 1).

Seed metabolic efficiency (SME) refers to the effectiveness of seed, where it converts stored nutrients into energy and biomass during germination and seedling growth. Besides, germination percentage and seedling length, SME is also an important indicator of plant growth. The combined application of microbes possessed the highest SME (3.33±0.07 g/g), followed by *Azotobacter* spp. (2.5±0.04 g/g), and *Rhizobium* spp. (1.17±0.12 g/g) than control SME (0.5±0.21 g/g) (Table 1). From the results it was observed that higher GP and SL occurs because of higher SME. The combination of the *Azotobacter* and *Rhizobium* spp. possess the highest SME value with significant difference compared to individual application of them and control.

The relative water content (RWC) of seedlings indicates the hydration level of the cells and often used as an indicator of plant stress. Biopriming with *Azotobacter* + *Rhizobium* spp. exhibited the maximum value of RWC (86.06±0.29%) followed by *Azotobacter* spp.(85.59±0.43%) and *Rhizobium* spp. alone (83.47±0.76%) compared to the control RWC (74.35±0.13%) (Table 1).

The plant-later stage growth (biomass, shoot and root length and number of leaves) parameters were also studied and significant differences were observed. The combined application of *Azotobacter* and *Rhizobium* lead to the maximum value of shoot length (58.8±0.12 cm), followed by the *Azotobacter* spp. (43.3±0.61 cm) and *Rhizobium* spp. (24.5±0.54 cm), than the value of control shoot length (14.6±0.32 cm) (Table 2 and Fig. 3). Similar to the shoot length, biopriming has been also found to enhance the root length of microbial treated seedlings compared to untreated control (Table 2 and Fig. 3).

The number of leaves is closely related to the plant growth and in particular to the shoot length, the larger size plant shoots led more number of nodal regions and in resultant develops more leaves. Biopriming with combination of *Azotobacter* spp. and *Rhizobium* spp. resulted in the maximum average number of leaves per plant (11 leaves/plant) followed by individual application of *Azotobacter* (10 leaves/plant) and *Rhizobium* (7 leaves/plant) compared to untreated control plants (5 leaves/plant) (Table 2 and Fig. 3).

Biomass of the plant is the indicator of level of physiological processes like photosynthetic activity, nutrient uptake and utilization, vigour and overall growth. Biopriming with combined application of *Azotobacter* + *Rhizobium* spp. has resulted maximum value of biomass (137.32±2.03 g) with significant difference compared to individual microbes and untreated control plants (Table 2).

DISCUSSION

Microbial treatment has an important role in increasing the seed germination, overall growth, and yield of a crop without using harsh chemicals [6]. Thus this chemical free approach of enhancing the growth and yield of the plant was studied. Various physico-chemical parameters were assessed and optimized in the curret study. The concentration of the bacterial culture was assessed and 10^8 CFU/mL concentration was considered as the optimal concentrations at which both GP and seedling growth were maximum. This observation was consistent with the findings in tomato, where *Trichoderma asperellum* showed the most significant result at 1×10^8 concentration [55]. According to Kerečki *et al.* (2021) [23] *Azotobacter* spp. at lower concentration (10^7 CFU/mL) has been shown to improve the GP and seedling growth in cucumber, tomato, wheat, soyabean, while no such effect was observed in white mustard and canola. This indicates that in Brassicaceae plant species, lower concentration of *Azotobacter* spp. is not effective in promoting seed germination and seedling growth that might be due to insufficient production of growth hormone required for biopriming. In a report it has observed that at higher microbial concentrations, phytohormone production was also higher which helps in germination and plant growth [25]. In the present study we also observed that lower concentrations were not efficient while at higher concentrations (10^8 and 10^9 CFU/mL) of *Azotobacter* spp. we found it promotive to GP and seedling growth.

Among the different temperatures checked, the optimized temperature was found to be 20°C where the maximum GP was obtained. The temperature was found to affect the biopriming significantly as evidenced in *Pennisetum glaucum* where *Azotobacter* spp. increases the plant growth at 20°C, and after that, an increase in temperature negatively affects the microbial growth and reduces its efficiency of nitrogen fixation [53] which corresponds to our study where at 20°C *Azotobacter* spp. resulted highest GP and seedling growth. Further, the lower temperatures considerably accelerate the growth, likely through lowering the competition for metabolic resources between bacteria and seeds. Also the duration of biopriming significantly affects the growth of the plant with 16 hours duration found the best for biopriing resulting in the maximum GP, ASL and ARL. According to Galindo *et al.* (2007) [16] and Kurdish *et al.* (2008) [26], the extended biopriming duration leads to excessive accumulation of extracellular polysaccharide by *Azotobacter* spp. which diminishes its adhesion efficiency to the seed surface and impairs key metabolic functions and thereby decreases the

effect of biopriming. Our study also indicates that extension of the biopriming duration over threshold duration negatively influences germination percentage (GP) and seedling growth.

The solution used for the biopriming also effects the process significantly. Different solutions were tested for the efficient biopriming and 1% NaCl solution gave the best response on GP, ASL and ARL. The results align with the studies in white mustard, cucumber, tomato, wheat, and soyabean where cells of *Azotobacter* spp. when suspended in NaCl solution showed highest GP and seedling length [23]. Similarly in case of tomato, when cells of other microbe like *Trichoderma harzianum* were suspended in NaCl solution showed an increase in GP, shoot and root length as compared to control [45]. Similarly the effect of pH of the solution on biopriming was accessed and the best response was observed on pH range of 5.5 to 7. Our finding was in correlation with the studies in canola and tomato where biopriming at pH 6 and 7.4 promoted the seedling growth [34, 45]. Ghotbzadeh and Gianinetti, (2018) [18] also reported that slightly acidic and neutral environments support the microbial activities while higher pH negatively influenced the biotic interactions which corresponds to our study where an increase in pH from 7 to 8.5 declines the biopriming response.

The above optimized parameters were applied to other bacterial strains and their combinations. The best response was obtained in the combined application of *Azotobacter* spp. and *Rhizobium* spp. resulting in highest GP. Consistent to our findings in other Brassicaceae crop canola, the combined application of PGPR like *Azotobacter* and *Azospirillum* species was shown to increases the growth and productivity [62]. The combined application of *Azotobacter* spp. and *Rhizobium* spp. was found to be the most effective in enhancing GP in different plant species such as *Trigonella foenum-graceum, Vicia faba* and *Zea mays* [12, 20, 37, 39]. Abdiev *et al.* (2019) [2] reported that *Azotobacter* and *Rhizobium* species alone or in combination enhanced the plant growth by adopting mechanisms like nitrogen fixation and uptake of nutrients from the soil, which could be the possible reason in our study also where these microbes individually or in combination enhanced the plant growth.

The effect of the application of the bacterial strains either alone or in combinations was tested on the GP, ARL and ASL to find out the SVI and SSI. The best response was obtained with the combined application of the two bacterial strains. Our results align with the report of Chaudhari *et al.* (2023) [11], where *Azotobacter* alone or combination increases the shoot length and root length of seedling as compared to control in *Heterophragma quadriloculare*. In *Eruca sativa* a Brassicaceae crop, *Rhizobium* species did not show significant difference in shoot and root length but showed proliferation in roots [49], though contrast to it in our study *Rhizobium* spp. has showed an increase in root length of the seedlings and showed significant difference compared to the control ARL. Our results also resembles with the canola and mustard, where treatment with *Azotobacter* enhances the vigor index [23].

Seed metabolic efficiency (SME), is another important plant growth parameter which measures the potential of the seed to convert stored food into energy. The highest SME was obtained in the combined application of the bacterial strains than the individual bacterial strain application. Similarly, RWC is an important factor related with the water availability in the seeds to carry out metabolic processes. The outcome of our study suggested that RWC has enhanced significantly by the biopriming treatment at the seedling stage with the combination of the two bacterial strains showing the best results. The results are in accordance with Dashadi *et al.* (2011) [12] where co-inoculation of *Azotobacter* and *Rhizobium* spp. also enhances the RWC. The possible reason for enhanced RWC could be due to the more uptake of water content because of good development of roots [3, 5].

The favorable outcomes observed in seed germination and seedling growth parameters due to biopriming treatments provide a strong foundation for anticipating positive impacts on plant-later stage growth (biomass, shoot and root length and number of leaves). The combination of the both bacterial starins during the biopriming resulted in the maximum shoot length obtained for the plant when compared with individual applications of bacterial strains and control. These findings were consistent with the studies in different crop plants where biopriming with individual or combination of microbes enhanced the shoot length such as in mustard (*Brassica Juncea* L.), radish, wheat and common bean [9, 33, 35, 56]. Similar results were obtained for the root length where the combination of both bacteria gave the best response. Hussein *et al.* (2016) [22] also reported that *Azotobacter* spp. promotes the root length in *Brassica napa* as compared to control, which is consistent to our study where *Azotobacter* spp. either alone or in combination increases the root length. Likewise, *Rhizobium* spp. either alone or in combination also enhanced the shoot and root length of *Brassica oleracea* var. kale, a non-legume crops. This promotive effect on the plant growth might be because of production of phytohormones (auxins such as indole-3-acetic acid and different cytokinins e.g. 2-isopentenyl adenosine, zeatine etc.) by the increased activity of *Rhizobium* spp. in response to plant root exudates [40]. Among the another possible reason, it was also reported that root exudates of *B. campestris*, supports the population growth of *Mesorhizobium* spp. that consequently enhances the nitrogen content [10].

The number and the area of leaves on a plant signifies its growth and its photosynthetic ability. The biopriming with the bacterial alone or their combination increased the number of leaves per plant

when compared to the control, with the combination being the most responsive. *Azotobacter* spp. alone or in combination promotes the leaf number, in accordance to Razmjooei *et al.* (2022) [48] this could happens due to the more uptake of nitrogen content, which ultimately promotes the biomass accumulation and leaf number and size. *Rhizobium* spp. resulted in fewer leaves compared to other treatments, the results have consistency with observation in *Sinapis alba* (Brassicaceae crops), where it was noticed that nodule formation activity of *Rhizobium* spp. has been affected that reduced nitrogen uptake from soil and could be the possible reason for lower number of leaves [21]. Biomass is a factor which is directly or indirectly related with the growth parameters of the plant. Here the biopriming also resulted in the increased biomass of the plant with combined application of both bacterial strains resulting in the highest biomass obtained. In various crops such as *Triticum aestivum*, *Cicer arietinum*, *Cajanus cajan*, *Helianthus annus*, and *Brassica nigra*, it was observed that *Azotobacter* spp. either alone or in combination showed an enormous positive response to plant characteristics such as increase in growth and nutrient uptake, which was corelated with our study where biopriming with *Azotobacter* spp. and *Rhizobium* spp. promoted the biomass [41, 43, 59].

In conclusion, the current study established the substantial consequences of microbial treatment on promoting seed germination, seedling length, vigor index, and development of plants by thoroughly evaluating the physico-chemical conditions managing seed biopriming in *Brassica oleracea* var. kale. The physico-chemical parameters for maximum seed germination and seedling growth were optimized with *Azotobacter* spp.at a concentration of 10^8 CFU/mL dissolved in a 1% saline solution at pH 7, and incubated for 16 hours at 20 °C. Biopriming with a combined application of *Azotobacter* spp. and *Rhizobium* spp. indicated a synergistic effect that delivered significant improvements across various parameters such as GP, ASL, ARL, SVI, RWC, and SME at the seedling stage in comparison to untreated control and individual treatment. In addition to improving growth at initial developmental stages, these microbial combinations have an immense effect on biomass, number of leaves, shoot length, and root length on mature plants, which ultimately increased the growth of *Brassica oleracea* var. kale. The findings ought to be validated in future studies using a wide range of cultivars and evaluation of agronomic parameters. It might encourage global attempts towards environment friendly farming by accelerating the advancement of sustainable biopriming technology.

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