



Effect of Bioinoculants and Plant Growth Regulators on Germination and Seedling Growth of Wild Ber (*Ziziphus rotundifolia* Lamk.) under *in-vitro* Conditions

Aayush Singla, Mukesh Kumar, Jeet Ram Sharma, Manish Kumar¹, Maya Lamba², Arjoo Dhundwal¹, Ankit Gavri and Mukesh Bishnoi

Department of Horticulture, Chaudhary Charan Singh Haryana Agricultural University, Hisar- 125 004, India

¹Department of Horticulture, Maharana Pratap Horticultural University, Karnal- 132 001, India

²Division of Fruits and Horticultural Technology, IARI, New Delhi- 110 012, India

E-mail: aayush.singla.750@gmail.com

Abstract: The prime motive of this experiment was to investigate the effectiveness of bioinoculants and plant growth regulators (PGRs) in improving the germination rate and seedling growth of wild ber seeds under *in-vitro* conditions. The experiment was conducted at the Department of SST, CCSHAU, Hisar, India, during the 2021-22 season. The seeds removed from stone were treated with five bioinoculants viz, Azoteeka- Mac 27, Azoteeka- HT 54, Phosphoteeka- P36, *Azospirillum*-J11-12 and integrated biofertilizer (NPK- 1:1:1) by soaking for 30 min and dipped in six-plant growth regulators (PGRs) viz., 50 mg/L gibberellic acid (GA₃), 100 mg/L GA₃, 50 mg/L 1-naphthalene acetic acid (NAA), 100 mg/L NAA, 50 mg/L indole-3-butyric acid (IBA), 100 mg/L IBA for 24 hrs and control. Among different treatments under laboratory conditions, maximum seed viability, maximum germination percentage, maximum vigour indices [vigour index- I, vigour index- II] and the least mean germination time were recorded when seeds were treated with GA₃ @100 mg/L for 24 hrs. Among different bioinoculant treatments, the seed treated with integrated biofertilizer (NPK- 1:1:1) for 30 min led to the highest seedling length, maximum seedling fresh weight and maximum seedling dry weight.

Keywords: Ber orchards, *In-vitro* conditions, Rhamnaceae, Rootstocks, Vigorous seedling

Ziziphus rotundifolia Lamk. (Wild ber), belongs to the family *Rhamnaceae*. It is one of the most important rootstocks for commercially grown ber orchards in India. It is native to an area that stretches from India to southwestern China and Malaya (Vavilov 1951). The area under ber cultivation in India is approximately 53,000 hectares with a production of 580,000 MT (2020-2021) and in Haryana, the area under ber cultivation is 4400 hectares with a production of 44,740 MT in 2020-21 (Anonymous 2020). *Ziziphus* is important underutilized fruit crop globally. Wild ber is frequently used as rootstock and hardy species against climate change. It is one of the hardest fruit trees and grows easily in a wide range of soils and climatic conditions. Currently, the area under cultivation of ber is continuously increasing but there is shortage of seedling availability. Improvement in the germination and production of vigorous seedlings is crucial for ber nursery growers. The ber rootstocks are prepared through seeds/ stones, as they are responsible for determining the quality of the orchard. Additionally, supplies water and nutrients to the plant and supports the plant, helping to regulate tree vigour, size and various other fruit characteristics. The ber rootstock produced from *Ziziphus rotundifolia* Lamk. is one of the

hardest rootstocks among ber species. Presently, rootstocks are prepared by sowing stones, but their germination is less uniform, poor and delayed due to the hard seed coat (endocarp). Seeds can be extracted from stones either mechanically or chemically but it might damage the embryo which results in irregular germination. The dormancy of seeds might be due to the hard seed coat which can be overcome to a greater extent by physical removal of seed coat or using pre-sowing treatments. The seed coat has an inhibitory role in gaseous exchange and seed germination.

Presowing treatments with bioinoculants and plant growth regulators (PGRs) play a significant role in seed germination and seedling growth of ber. Several fruit crops showed spectacular results from the use of PGRs in terms of germination, growth, yield, and quality. PGRs such as gibberellic acid (GA₃), 1-naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA) enhance the germination, growth and survival ability of seedlings (Patel 2019). GA₃ is used to weaken seed coat dormancy so that the radicle of the seedling can break through the seed coat. It induces the synthesis of amylase and other hydrolytic enzymes during the early stages of seed germination and controls the mobilization of starch which acts as a respiratory substrate

leading to immediate enhancement in cell elongation. GA₃ also helps in enhance the availability of reserved mineral elements that promote the germination process. The seeds soaked in GA₃, NAA and IBA for 24 hours resulted in higher germination (Hakimi 2020). Scarification of ber seeds using 250 mg/L GA₃ for 24 hrs performed best in terms of germination and seedling growth under lab conditions (Sheoran et al 2019).

In recent years, bioinoculants have been used to suppress plant diseases (Heitefuss 2001) and nitrogen mobilization in soil (Saba et al 2012). They are less expensive alternatives to PGRs and boost productivity in short duration by enhancing the soil fertility and favouring phytopathogenic organism antagonism and biological control (Chirinos et al 2006). Bioinoculants stimulated seed germination compared to the control. The mechanism of action of plant growth-promoting rhizobacteria (PGPR) consists of enhancing the seed germination process and excreting phytohormones such as auxin and gibberellins. These factors improve seed germination and early seedling development. In addition to metabolic activities, bacteria excrete organic acids which help in nutrient uptake at later stages of growth (Bakonyi et al 2013). Inoculation with *Azotobacter* resulted in the earliest germination (14 days), 50 percent germination (17 days) and maximum germination percentage (82%) over the control (Subhashlal 2017). The prime motive of this experiment was to investigate the effectiveness of bioinoculants and plant growth regulators (PGRs) in improving the germination rate and seedling growth of wild ber seeds under *in-vitro* conditions.

MATERIAL AND METHODS

Experimental site: The present study on the effect of bioinoculants and PGRs on the germination and seedling growth of wild ber seeds was carried out at the Laboratory of Department of Seed Science and Technology (Location: 29°9'14.838" N, 75°42'8.178"E), CCS Haryana Agricultural University, Hisar during 2021-22.

Planting material: The ripened fruits of *Ziziphus rotundifolia* were collected from the fruit orchard of the university (Location: 29°9'17.859" N, 75°41'40.238 E"). The fruits were dipped in water tank to soften the pulp for stone removal. After that the stones were dipped in a water tank for 2-3 days and then shade dried to extract seeds mechanically from stones. All methods performed in this study involving the use of *Ziziphus rotundifolia* ripened fruits were conducted in accordance with the relevant institutional, national, and international guidelines, regulations, and legislation

Seed treatment: The seeds were treated with PGRs at concentrations of 50 mg/L and 100 mg/L each. PGRs were

first dissolved in 95% ethyl alcohol or sodium hydroxide (NaOH). Then, a solution of 500 ml of each PGR was prepared. The seeds were soaked in the prepared solution for 24 hrs and then shade dried. For bioinoculants treatment, the seeds were first dipped in jaggery solution. Thereafter, the samples were mixed thoroughly with different bioinoculants each of concentration 1×10^8 CFU in a beaker for 30 minutes and shade dried. All the treatments of bioinoculants and PGRs were applied separately.

Treatment details: Twelve treatments with three replications each were selected based on previous literature. 75 seeds per replication were sown for the experiment. In addition, the formulations of bioinoculants with specific concentrations (T2 to T5) were grown in the laboratory of microbiology, CCSHAU, Hisar, India- 125004. Formulation T6 was collected from the Indian Agricultural Research Institute, New Delhi. After treatment the seeds were dried in the shade and then sown in between paper method.

Parameters Determined

Seed viability: Twenty-five seeds per replication were soaked in 50 mL water for 16 hours at 25°C to activate dehydrogenase enzymes. Seeds were di-sectioned longitudinally with a sharp blade and stained in 0.01 percent tetrazolium solution for 5 hours at room temperature in petri dishes. The seeds were rinsed in running tap water and seed viability was observed with the naked eye/ convex lens as a change in colour (pink). The germination parameters were evaluated after 24 hrs.

Germination percentage (GP): The germination percentage of the seeds was observed by keeping the treated seeds in between moist paper and keeping them in an incubator at a temperature of 25±2°C with 80-85% relative humidity. Radicle appearance was observed with an interval of every 24 hrs regularly up to 21 days after sowing.

$$\text{Germination (\%)} = \frac{\text{Total number of seeds germinated}}{\text{Total number of seed sown}} \times 100$$

Mean germination time (MGT) (days): Mean germination time (MGT) (days) was calculated by counting the normal germinated seeds (growth was normal after germination). MGT was considered to be when most of the seeds germinated. The germination count was taken at 24-hour interval for regular 21 days after sowing and calculated by the following formulae:

$$\text{MGT} = \frac{\sum nd}{\sum n}$$

where,

n = number of seeds that germinated on day d, and

d = number of days counted from the beginning of the germination test.

Seedling length (cm): Ten randomly selected normal seedlings per replication were taken for seedling length. It was measured from the root tip to the shoot tip with the help of a meter rod. Average seedling length was expressed in cm.

Fresh weight per seedling (mg): Ten randomly selected normal fresh seedlings were weighed.

Dry weight per seedling (mg): Ten randomly selected normal seedlings were kept in a hot air oven drier at $60 \pm 2^\circ\text{C}$ until a constant weight was achieved. Dried weight of seedling was determined.

Seedling vigor: Seedling vigour indices I and II were calculated (Abdul baki and Anderson 1973)

Vigor index-I = Standard germination (%) \times Average seedling length (cm)

Vigor index-II = Standard germination (%) \times Average seedling dry weight (mg)

Correlation coefficients: Among all possible character combinations at the phenotypic 'r (p)' level were estimated (Al-Jibouri et al 1958).

$$\text{Phenotypic correlation } r_{xy}(P) = \frac{\text{Cov}_{xy}(P)}{\sqrt{V_x(P) \times V_y(P)}}$$

where, $\text{Cov}_{xy}(P)$ = Phenotypic covariance between characters 'x' and 'y'

$V_x(P)$ = Phenotypic variance of character 'x'

$V_y(P)$ = Phenotypic variance of character 'y'

Path coefficient analysis: This was performed as per Wright (1921) and adopted by Dewey and Lu (1959). Standard path coefficients, also referred to as the standardized partial regression coefficients, were calculated. These values were obtained by solving the following set of 'p' simultaneous equations using INDOSTAT software. This method was used to determine the direct and indirect paths.

Principal component analysis: PCA was performed to analyze complex datasets and is based on either correlation or variance-covariance matrices. Standardizing variables can address scale effects, especially when variables are measured differently. Correlation matrices are often preferred for extracting principal components in modern analyses.

Statistical analysis: The were statistically analysed using SPSS 16.0C.

RESULTS AND DISCUSSION

Seed viability and germination percentage: The seed viability was significantly influenced. The maximum seed viability of ber was recorded in 100 mg/L GA_3 followed by phosphoteeka, *Azospirillum* J 11-12, integrated biofertilizer (NPK- 1:1:1), 50 mg/L GA_3 and 100 mg/L IBA, was 92%, which was higher than that of all other treatments. The germination percentage (GP) was significantly influenced by

different presowing treatments with PGRs and bioinoculants (Table 1). GP was significantly increased with different presowing treatments when compared to the control. GP ranged from 54.22 to among different treatments. The maximum GP of ber seeds was in 100 mg/L GA_3 for 24 hours (80.44%) which was statistically at par with 50 mg/L GA_3 and integrated biofertilizer (NPK- 1:1:1). The minimum germination percentage (54.22%) was in control, which was statistically at par with 50 mg/L IBA and *Azospirillum* J 11-12. The GP with GA_3 increased because it acts as a growth regulator for breaking seed dormancy (Koyuncu 2005). It plays an important role in the germination of seeds by leaching out retardants. GA_3 aids the aleurone layer of the seed during the synthesis of hydrolytic enzymes that stimulate the α -amylase enzyme that converts insoluble sugar into soluble sugar (Babu et al 2010). GA_3 stimulates the cell wall to release and transmit its calcium into the cytoplasm. It affects the enzyme synthesis that produces mRNA and thereby increases DNA replication and induces analysis of endospermic materials in the seed (Lahuti et al 2003). The possible mechanism of PGPR in the seed germination process is based on the fact that bacteria can excrete phytohormones such as auxin and gibberellic acid and thereby, improve seed germination and early seedling development. Joshi et al. (2015) also reported that the germination of acid lime seeds was significantly influenced by 200 mg/L GA_3 . Similar findings were reported by Karimpour et al. (2013). Hence, GA_3 increased the rate of reactions responsible for seed germination.

Mean germination time: The minimum MGT (9.7 days) was in 100 mg/L GA_3 , which was statistically at par with *Azospirillum* J 11-12, 50 mg/L GA_3 , Azoteeka- HT 54, 50 mg/L NAA, integrated biofertilizer (NPK- 1:1:1) and phosphoteeka. However, maximum MGT of 11.1 days was in control, which was statistically at par with Azoteeka- Mac 27 (Table 1). Early seed germination with GA_3 treatment might be due to stimulation of the α -amylase enzyme that converts insoluble sugar into soluble sugar, which provides energy for early seed germination. Sheoran et al. (2019) also reported that MGT was significantly influenced by 250 mg/L GA_3 for 24 hrs. Bioinoculants also help in early germination because the bacteria excrete phytohormones such as auxin and gibberellins, which are helpful to reduce MGT.

Seedling length (cm): The results reveal that seedling length was significantly increased by all pre-sowing seed treatments in comparison to the control. The maximum seedling length was in integrated biofertilizer (NPK- 1:1:1) (20.33 cm), which was statistically at par with 100 mg/L GA_3 and 50 mg/L GA_3 whereas, minimum seedling length (8.7 cm) was in control (Table 2). higher seedling length with

integrated biofertilizer may be due to sufficient availability of nitrogen, phosphorus, potassium and other essential nutrients. The optimum supply of plant nutrients in the right amount at right time *i.e.*, during the vegetative growth period induce vegetative growth which ultimately increases seedling length. The higher seedling length with GA₃ may be due to the overall growth of seedlings and enhanced rate of photosynthesis and translocation of photosynthates in seedlings. Govind (2021) also reported that gibberellic acid significantly increased seedling length in bael and Hakimi (2020) reported the same in avocado.

Fresh and dry weight per seedling (mg): The fresh and dry weight per seedling was significantly increased by all pre-sowing seed treatments in comparison to the control (Table 2). The maximum fresh weight per seedling was in integrated biofertilizer (NPK- 1:1:1) (159.8 mg) which was statistically at par with 100 mg/L GA₃, 50 mg/L GA₃, Azoteeka-HT 54, 100 mg/L NAA and 50 mg/L NAA whereas, minimum fresh weight per seedling (123.3 mg) was in control. Dry weight per seedling at 21 DAS was maximum with in

integrated biofertilizer (NPK- 1:1:1) (35.06 mg), which was statistically at par with 100 mg/L GA₃ and 50 mg/L GA₃. The lowest dry weight per seedling (25.51 mg) was recorded for the control, which was statistically at par with the *Azospirillum* J 11-12, 50 mg/L IBA, 100 mg/L IBA, Azoteeka- Mac 27. The reason for higher fresh and dry weight per seedling with integrated biofertilizer may be due to sufficient availability of nitrogen, phosphorus, potassium and other essential nutrients. The optimum supply of plants nutrients in the right amount at the right time *i.e.*, during the vegetative growth period induce vegetative growth which ultimately increased seedling length and dry weight per seedling. The higher fresh and dry weight per seedling with GA₃ may be due to the overall growth of seedlings and enhanced rate of photosynthesis and translocation of photosynthates in seedlings. Kumar et al. (2020) also reported that fresh and dry weight of roots and shoots increased significantly by help of gibberellic acid in ber and Gupta et al (2018) reported the same in guava using biofertilizers.

Vigor indices: The vigor indices I and II were significantly

Table 1. Effect of bioinoculants and plant growth regulators on seed growth parameters of *Ziziphus rotundifolia*

Treatments	Seed viability (%)	Germination (%) 21 DAS	Mean germination time (days)	Seedling length (cm) 21 DAS	Fresh weight per seedling (mg) 21 DAS	Dry weight per seedling (mg) 21 DAS
Control (Untreated)	80	54.22	11.1	8.7	123.3	25.51
Azoteeka (<i>Azotobacter chroococcum</i> , Mac 27)	80	61.78	11	16.57	143.4	27.39
Azoteeka (<i>A. Chroococcum</i> , HT 54)	84	76	9.9	17.23	156.7	31.47
Phosphoteeka (<i>Pseudomonas</i> , P 36)	92	67.56	10.1	16.83	148.8	28.29
<i>Azospirillum</i> (J 11-12)	92	57.33	9.8	12.43	129.5	26.31
Integrated biofertilizer (NPK-1:1:1)	92	77.78	10.1	20.33	159.8	35.06
50 mg/L GA ₃	92	79.56	9.8	19.9	158.1	33.59
100 mg/L GA ₃	92	80.44	9.7	20.3	158.2	34.33
50 mg/L NAA	80	71.56	10	16.83	154.7	29.73
100 mg/L NAA	80	74.22	10.1	17.43	156.3	31
50 mg/L IBA	80	56.44	10.2	14.23	139.1	26.47
100 mg/L IBA	92	62.22	10.3	15.73	140.2	27.27
CD (p=0.05)	6.8	3.64	0.4	1.81	7.6	2.55

Table 2. Path coefficient analysis showing direct (Diagonal and bold) and indirect effects on component traits on dry weight per seedling

Variable	Seed viability	Germination percentage	MGT	Seedling length	Fresh weight per seedling	Dry weight per seedling
Seed viability	0.066	0.253	-0.029	0.081	-0.029	0.343
Germination percentage	0.019	0.881	-0.033	0.199	-0.169	0.898
MGT	-0.033	-0.492	0.058	-0.118	0.095	-0.489
Seedling length	0.024	0.772	-0.030	0.228	-0.173	0.820
Fresh weight per seedling	0.010	0.779	-0.029	0.206	-0.191	0.774

Residual effect= 0.4195

increased in presowing seed treatments as compared to the control (Fig. 1). The maximum vigour index I (1633) was d in 100 mg/L GA₃, which was statistically at par with 50 mg/L GA₃ and integrated biofertilizer (NPK- 1:1:1). However, the lowest vigour index I (472) was in control. The maximum value (2762) of vigour index- II was in 100 mg/L GA₃, which was statistically at par with integrated biofertilizer (NPK- 1:1:1) and 50 mg/L GA₃. However, the lowest vigour index- II (1383) was reported for the control, which were significantly at par with the 50 mg/L IBA and *Azospirillum* J 11-12. In the current study, GA₃ increased seedling length indicated in vigour index I. The vigour index II is based on the rate of dry matter accumulation and both indicates the availability of reserve food material and the termination of seed dormancy. The greater vigour index- II indicates more vigorous seed. The seedling length decreases as the spacing decrease, while the dry matter accumulation increases as the spacing increases. Both indices were tested in the laboratory and showed that the ber seed treated with 100 mg/L GA₃ had longer seedling length and greater dry matter accumulation. Ashish (2021) reported that vigour- I and vigour- II were found significantly better by help of gibberellic acid in papaya and also, Yadav (2021) reported the same in jatti khatti.

Correlation: The dry weight per seedling was the dependent variable among all the traits. The dry weight per seedling was positively and significantly correlated with seed viability (0.343), GP (0.898), seedling length (0.820) and fresh weight per seedling (0.774) whereas, negatively and significantly correlated with MGT (0.489) (Fig. 2).

Path coefficient analysis: Using dry weight per seedling as a dependent variable and the rest of the traits as independent variables, the path coefficient analysis was used to evaluate the positive and negative, direct and indirect effects of different traits on dry weight (Table 3). The examination of

path coefficient analysis indicated that maximum direct positive on dry weight per seedling was imposed by GP (0.881) followed by seedling length, seed viability and MGT whereas, the maximum indirect positive effect was exhibited by fresh weight per seedling through GP (0.779) followed by seedling length through GP, seed viability through GP and fresh weight per seedling through seedling length. The residual effect at phenotypic level was 0.4195 which means there are some other traits or factors that affect dry weight per seedling.

Principal component analysis (PCA): Principal component analysis (PCA) condenses multiple variables into a few independent components, revealing their relative importance. In this study, PCA was conducted using the Sneath and Sokal method (1973), considering components with eigenvalues >1 (Kaiser 1958). Eigenvalues determined the number of factors retained, with the total variables equating to the sum of eigenvalues. The first two principal

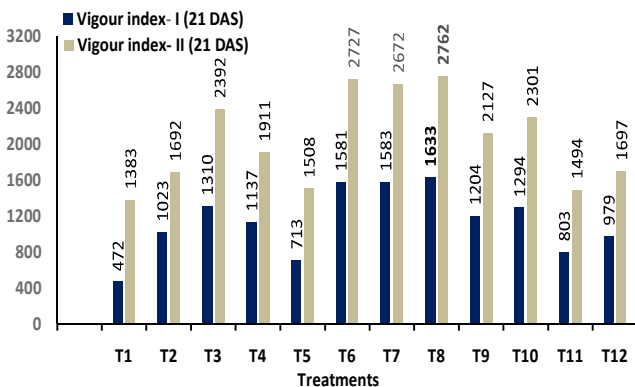
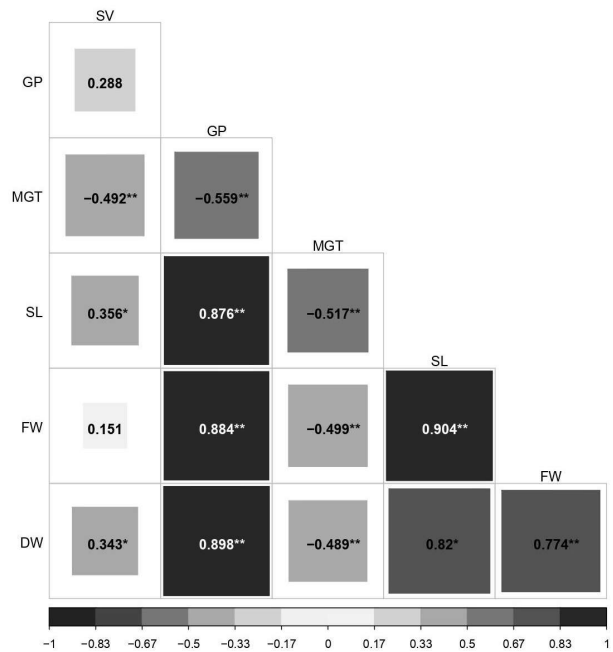


Fig. 1. Effect of bioinoculants and plant growth regulators on Vigour index- I and Vigour index- II of *Ziziphus rotundifolia* seeds when utilizing the in between paper method



*Significant at 5% level; **Significant at 1% level; SV, Seed viability; GP, Germination percentage; MGT, Mean germination time; SL, Seedling length; FW, Fresh weight of seedling; DW, Dry weight of seedling

Fig. 2. Phenotypic coefficients of correlation among different traits

Table 3. Total variance explained by different principal components in treatments of wild ber

Principal component	Eigen value	Percentage of variance	Cumulative percentage of variance
PC1	4.38	72.993	72.993
PC2	1.014	16.896	89.889

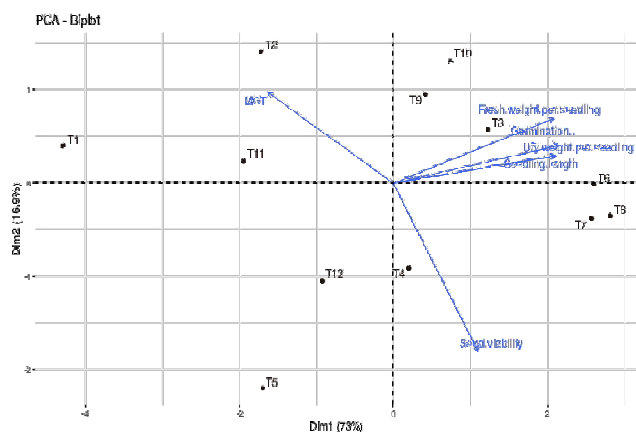


Fig. 3. Principal component loading plot of PC-1 and PC-2

components (PC 1 and PC 2) had eigen value greater than one and they cumulatively explained 89.889% of the total variation present in original data set (Table 3). The first and second principal components explained 72.993 and 16.896 percent of the total variability, respectively.

The first principal factor showed high positive loading for germination percentage (0.461), dry weight per seedling (0.453), seedling length (0.451) and fresh weight per seedling (0.447). Principal factor two enabled high positive loading for seed viability (0.803). The PCA of the treatments is described in terms of spatial distance (Fig. 3). The biplot of genotypes based on PC-1 and PC-2 depicted 89.89% of total variation. The vector of traits, viz. dry weight per seedling, fresh weight per seedling and seed viability showed longer lengths, indicating positive contribution to both components.

The treatments of bioinoculants and PGRs have been distributed on the basis of their relative performance with respect to principal factor one and two. Principal factor one showed high loading for germination percentage, dry weight per seedling, seedling length and fresh weight per seedling. Therefore, treatments (T1, T2, T3, T6, T9, T10, T11) lying on the positive side of PF-1 show high variability for these particular traits. Principal factor two showed high loading for seed viability. The treatments (T3, T4, T6, T7, T8, T9, T10) lying on the positive side of PF-2 show high variability for these particular traits. In conclusion for both PC, treatments (T3, T6, T9, T10) lying on positive side of both PC shows high variability for germination percentage, seedling length, fresh weight per seedling and dry weight per seedling.

CONCLUSION

Different presowing treatments had a significant effect on improving seed germination and seedling growth of species *Ziziphus rotundifolia* compared to the control. The experiment showed that treating wild ber seeds with 100 mg/L gibberellic acid (GA_3) for 24 hours significantly

improved seed viability, germination rate and vigour indices. Additionally, applying integrated biofertilizer (NPK- 1:1:1) for 30 minutes resulted in enhanced seedling growth in terms of length, fresh weight and dry weight. These findings underscore the efficacy of GA_3 and integrated biofertilizer in enhancing both seed germination and seedling growth, providing valuable insights for optimizing wild ber cultivation practices in controlled environments.

AUTHORS CONTRIBUTION

AS, MK^a and JRS conceived the study, designed, analysed data and interpreted the results of the experiment. AS wrote the first draft of the paper. AS, MK^a and MK^b cowrote and edited the manuscript. AS, MK^a, ML and AD managed the literature search. AS, MK^a, AG and MB critically revised the manuscript. All authors reviewed the manuscript and finalized it.

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