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BamFx Induced Resistance by Seed Priming Method against *Fusarium* sp. in *Cajanus cajan* (L.) Mill sp.

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ABSTRACT

Background: Pigeon pea [*Cajanus cajan* (L.) Mill sp.] enriches the soil through symbiotic nitrogen fixation. The pigeon pea plants are susceptible to a number of pathogenic fungi including *Aspergillus* sp. and *Fusarium* sp. Bam-FX® is a micromineral formulation that directly affects the physiological mechanisms and innate immune system of plants to help them overcome both abiotic and biotic stresses without the need for any form of genetic modification.

Methods: In this study, we have tested different dilutions to soak the pigeon pea seeds. On 21th day, optimum germination (94%) was found in seeds treated with BamFX (1:1000) compared to 75% germination in untreated seeds.

Result: The fungi were isolated from the soil and identified using 18s rRNA gene sequencing. The seedlings in BamFx treated groups were found resistant to pathogenic fungi, whereas, more than 97% in the untreated groups were found infected or diseased. The increased germination efficiency and resistance to fungal infections in BamFx treated seedlings proved a priming effect due to BamFx in pigeon pea seeds.

Key words: *Aspergillus* sp, BamFx, Collar rot, *Fusarium* sp, Pigeon pea.

INTRODUCTION

Pigeon pea [*Cajanus Cajan* (L.) Mill sp.], is a perennial tropical legume that involved in nitrogen fixation in soil (Kharte *et al*., 2022; Bohra *et al*., 2020; Nisha *et al*., 2021; Talari *et al*., 2018; Patil *et al*., 2017). It is one of the most significant crops in India after chickpea, dominating an area of 4.89 Million Hectares, with a productivity of 762.4 kilograms per hectare (Sanap *et al*., 2016 and Bohra *et al*., 2020). Although it is the most widely used crop, it suffers from significant environmental problems (abiotic and biotic stress). Low and high moisture, salinity and waterlogging hypoxia are a few examples of stressors that affect production (Zavinon *et al*., 2020). The annual loss of crop due to wilt in India alone has been estimated at US $71 million (Ayenan *et al*., 2017; Patil *et al*., 2017). Previous reports have indicated contamination of pigeon pea collected in southern Benin by fungi with a high occurrence of *Aspergillus* strains (71.42%) (Sultan and Magan, 2010). This contamination is characterized by the presence of *Aspergillus parasiticus*, *A. flavus and A. ochraceus*. This high contamination by *Aspergillus* strains could be in relation with the type of fungi present in the soil of culture areas (Barros *et al*., 2003). In other report, *Rhizobium* spp. was isolated from root nodules of pigeon pea (Nushair *et al*., 2018). Previous reports on the toxinogenic potential of these fungi involved peanut cakes (S. Adjou, 2012), as well as peanut seeds gathered from Benin (Boli *et al*., 2020). When food storage conditions are poor, these fungi can create a considerable amount of mycotoxins (Boli *et al*., 2020).

Application of fungicides, bioagents and phytoextracts in seeds can reduce disease, increase genetic potential and ultimately increase output. These fungicides are toxic in nature and responsible for various diseases in the human population. Bam-FX® is a proprietary elicitor composition developed by Zero Gravity Solutions Inc. It directly affects the physiological mechanisms and innate immune system of plants to help them overcome both abiotic and biotic stresses without the need for any form of genetic modification. It helps in reducing the pest and microbial infestations and in obtaining better yield even in regions with cold, heat, or water stress. It enhances nutrient absorption, chlorophyll content and antioxidant activity and induces systemic responses within the plant body. In this study, we have used different dilutions to soak the pigeon pea seeds for 30 mins. Fungi were isolated from the soil and identified using 18 s rRNA gene sequencing. The effect of BamFX on the growth and development of pigeon pea seedlings in presence of fungal pathogens was observed.

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MATERIALS AND METHODS

BamFx composition and preparation of working solution

Copper 2% (w/v) and zinc (7% w/v) mixed in water and pH was adjusted to 1.5 with H_2SO_4. BamFx was diluted to 1:300, 1:700,1:1000, 1:1500 and 1:2000 in water for further experiments. The work was carried out in MIT Aurangabad, India during 2020-2022.

Pigeon pea seed treatment with BamFx solutions

Pigeon pea seeds were taken in different Petri dishes. The seeds were soaked in BamFX 1:300 to 1:2000 dilutions for 30 min. After 30 min soaking in the BamFX dilutions, seeds were removed from the plate and sowed in the greenhouse under controlled conditions. One group was kept as untreated control.

Pigeon pea cultivation in fungus infected soil

After treating the seeds with BamFx solutions, the seeds were sown in soil. Then it was observed and data were recorded after every 24 hours for germination and different growth parameters.

Identification of fungal diversity in the soil

The soil (1 g) was mixed with 10 ml of sterile distilled water. After mixing for 5 min, serial dilutions up to 10^{-4} were made in different test tubes. The diluted suspensions 50 µl were spread on potato dextrose agar plates and incubated for 96 hours at 28°C. The fungal colonies were isolated from the plates and preserved on agar slants. The fungus inoculated in potato dextrose broth (pH7.2) and incubated at 28°C for 48 hours. The fungal culture was used for further experimentation.

Isolation of genomic DNA and amplification of the 18S rDNA region

The DNA sequencing method was used for the sequencing of 18S rDNA of fungal cultures. The procedure used is described previously (Kharat *et al*., 2010). Mid-log phase cultures of selected isolates were used for PCR amplification of the 18S rDNA region. Cultures were centrifuged at 6000x gravity and washed twice before DNA isolation. Total genomic DNA isolation was carried out with a kit from Life Technol. Inc. USA. Isolated and purified genomic DNA was used as a template for PCR amplification. The PCR program used was as follows: 95°C for 2 min followed by 29 cycles of 94°C for 30 sec, 52°C for 30 sec, 72°C for 1 min, the final extension step at 72°C for 5 min was carried out for the maturation of the amplicon. Purified amplicons were then used in deciphering 18S rDNA nucleotide sequence.

DNA sequencing

Forward and reverse DNA sequencing reactions were carried out using the BDT v3.1 cycle sequencing kit on ABI 3730xl genetic analyzer at Eurofins lab (India).

DNA sequence data analysis

The 18S DNA sequences were analysed initially using the BLASTn search with http://www.ncbi.nlm.nih.gov/blast search engine. CHECK_CHIMERA program of http://www.ncbi.nlm.nih.gov/blast/ blast.cgi was used for the chimeric artifacts. The nucleotide information obtained from this endeavour was then deposited with Genbank. Data Analysis and Phylogenetic Tree Construction. All of the 18S rDNA sequences were also analysed using BLASTn search engine at http://www.ncbi.nlm.nih.gov/blast. The evolutionary history for these nucleotide sequences was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). To represent the evolutionary history of the taxa analysed, the bootstrap consensus tree inferred from 1000 replicates was taken (Felsenstein, 1985). The evolutionary distance was computed using the Kimura 2-parameter methodand are in units of the number of base substitutions per site. Phylogenetic analyses were conducted using MEGA4 (Tamura *et al*., 2007).

Statistics

Data are reported as mean±S.D. All experiments were done at least three times and three or more independent observations were made on each occasion. Statistically significant values were compared using one way analysis of variance (ANOVA) and p-values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

A pathogen that causes illness in numerous commercially significant crops is *Fusarium* sp. In this work, the fusarium infection in pigeon pea plants was managed using seed priming with BamFx. Even though *F. oxysporum* is being controlled using a variety of techniques (Naznin *et al*., 2014), the fungus's persistence and diversity make it extremely difficult to completely eradicate. Since F. oxysporum is a soil- and seed-borne fungus, it is exceedingly difficult and unsustainable to apply fungicide to soil. When agrochemicals are used excessively, it can cause problems for the environment and human health (Khan and Hanjra, 2008).

Effect of BamFX on seed infecting fungi and seed health

Within a month of sowing, a seedling disease typically manifests itself as patches of dead seedlings in the primary leaf stage that are dispersed over the field (Fig 1). Before they perish, the seedlings could become a little chlorotic.

Identification of fungus

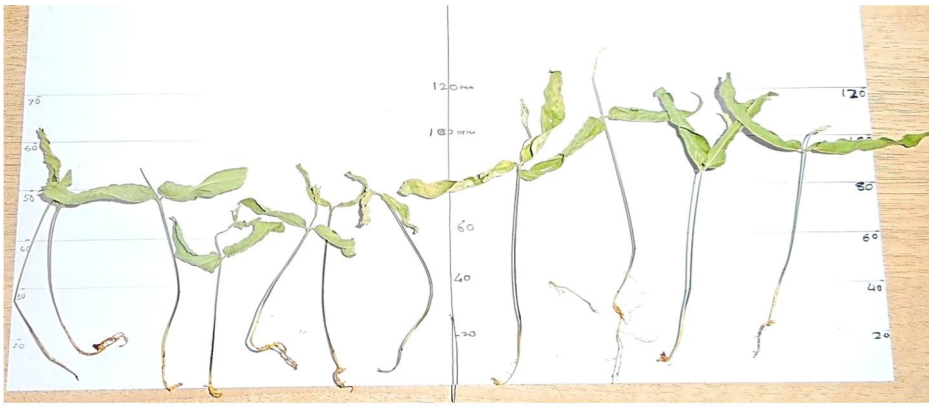
Thirty-nine (39) out of 40 control seeds, after attaining height of 50 to 60 mm were found to be uprooted and lying on the soil bed. The Plantlets were falling down on ground after reaching a height of 50 to 60 mm. The stem collar was the infected area not holding the plantlets in straight position. The control untreated plantlets were found lying on the ground and died. Another factor was that the lower part of the roots remained in the soil. Then, we started analysis of the microbial flora of soil where pigeon pea seeds were sown. Pigeon pea samples collected from the tested locations were found to be heavily contaminated by fungi, as determined by the results of microbiological

investigation and the isolation of fungi in pure culture. By analysis of 18s rRNA sequences , we have identified three fungal species , mainly-*Aspergillus ochraceus* (23.8%), *Fusarium oxysporum* (19.04%) and *Aspergillus flavus* (16.66%). *Fusarium* (26.19%) and *Aspergillus* (71.42%) were the two most frequent genera. The DNA sequences of the 18 S rRNA also proved the species identification. The Sequences were deposited to GenBank (GenBank IDs-ON597492.1, ON597493.1, ON597494.1, ON597495.1, ON597496.1).

Development of resistance against the fungal seedling rot

The BamFX-treated seedlings were found to have no symptoms of disease. The BamFX priming effect induced the resistance to fungal infection in the treated pigeon pea seedlings.

The BamFX-treated seeds germinated faster than the untreated seeds. The concentration of BamFx also affected the germination rate of the seeds. The 1:300 and 1:700 dilutions increased the germination of seeds from 28% (Untreated control) to 51% (1:300 dilution) and 60 % (1:700 dilution). On the 15th day, the optimum germination (70%) was observed in seeds treated with BamFx (1:1000) (Table 1). This increased up to 94% at 21 days and finally 100% seeds germinated after 23 days. The germination rate was 75% after 23 days in untreated seeds. There were fewer diseased or infected seedlings in the BamFx treated groups. In 1:300, 1:1,000 and 1: 1500 concentrations of BamFX, the number of diseased plants observed were 1,1 and 3, respectively. More than 97% of the plants were infected or diseased in untreated control seeds (Table1). The enriched GO keywords in our previously published work indicated that the DEGs were linked to signal transmission, stress response and enzymatic regulation of metabolism. BamFX changed the transcription of genes that control key processes, such as cell division, the cell cycle and regular metabolic patterns. By upregulating the expression of genes involved in the active regulation of oxidative stress in plants, BamFX had an impact on the antioxidant defence system. The stimulation of transcription factors and enhanced signal proteins was demonstrated by the differential gene expression in seedlings treated with BamFX. The seedlings treated with BamFX exhibited elevated levels of oxidative stress genes, defence protein and hormones (Kharat and Pottathil, 2022).Similarly, in this study, the increased germination efficiency and resistance to fungal infections in BamFX treated seedlings supports the theory that BamFX promotes a priming effect in pigeon pea seeds.



A B

(A) is the untreated control seedlings of *C. cajan*, (B) The BamFx treated seedlings of *C. cajan.*

Fig 1: The effect of BamFx on the *Cajanas cajan*.

Table 1: Germination of pigeon pea seeds in greenhouse soil.

BAM-FX dilutions of original stock solution	Total number of seeds used	Number of seed germinated on Day 15	Number of seed germinated on Day 21	Number of seed germinated on Day 23	Diseased plants	Percentage of diseased plants
300	80	41	55	62	1	1.61
700	80	48	68	75	16	21.33
1000	80	56	75	80	1	1.25
1500	80	41	66	70	3	4.28
2000	80	34	67	70	19	27.14
Control	40	11	26	30	29	96.66

The BamFX treated seedlings were found longer than the untreated control seedlings (Fig 2). The height, leaves and stem appeared healthy in BamFX treated seedlings compared to the untreated control group (Fig 1 and 2). The roots of the BamFX treated seeds were longer with secondary roots. The roots of the untreated group were short and bulb-shaped. The round bulb like structures in the untreated control seedlings was observed. There were brown sclerotial bodies of the fungus found attached to the collar region of a dead seedlings (Fig 3). The root tips were found yellow to dark brown in colour.

The collar region of the stem and roots were very weak and infected by fungi. The stem and roots had a large number of dark brown bulb like structures (Fig 3). The major infection was observed in the region of the upper part of the roots and the lower part of the stem. There were small white

B, C, D- The roots of the untreated group were short and had bulb-like shapes on the main roots.
A and E- No round bulb like structures of the untreated control seedlings observed.

Fig 2: The control untreated seedlings infected with the *Fusarium* sp.

A) Underground portion of the *Fusarium* sp. infected seedlings of *C. cajan.* B) Stem and root region of the *Aspergillus sp.* infected seedlings of *C. cajan.* C) Stem and root region of *Fusarium sp.* Infected seedlings of *C. cajan.*

Fig 3: The diseased roots and stem region of the control untreated seedlings of *C. cajan.*

hairlike structures on the diseased roots. These structures were not found in the BamFX treated seedlings. This confirms the priming effect of BamFx on the pigeon pea seedlings.

CONCLUSION

The study is the investigation of the BamFx for induction of priming in Cajanas cajan seeds. The priming was induced in the seeds and the disease resistance was developed against fungi A*spergillus ochraceus, Fusarium oxysporum and Aspergillus flavus*. The BamFX-treated seedlings were found to be longer and healthier than the untreated control group. The results suggest that the use of BamFX treatment can effectively improve seed health and increase the resistance of pigeon pea seedlings to fungal infections. Using the sources mentioned, it is clear that BamFX treatments can be applied to seeds in order to improve their health and increase resistance to diseases.

Conflict of interest

All authors declare that they have no conflicts of interest.

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