Middle East Journal of Agriculture Research
Volume: 13 | Issue: 01| Jan. – Mar.| 2024

EISSN: 2706-7955 ISSN: 2077-4605
DOI: 10.36632/mejar/2024.13.1.13
Journal homepage: www.curresweb.com
Pages: 223-233

Evaluation of Solid Organic Substrates Formulation on Viability and aspects of *Trichoderma asperellum*

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Received: 15 Jan. 2024 **Accepted:** 10 March 2024 **Published:** 30 March 2024

ABSTRACT

Trichoderma has picked up promising consideration as plant growth promoting fungi (PGPF). The display ponder was attempted to discover the foremost reasonable organic substrates for the biomass production of *Trichoderma asperellum* OR234761. The shelf life and viability of the *Trichoderma asperellum* were determined as colony-forming units per gram (CFUs g^{-1}) in each substrate through storing periods extended to one year at room temperature 25ºC and within the fridge 4ºC. The results showed that *Trichoderma asperellum* OR234761colonized about all substrates after 21 days of incubation with diverse degree of colonization which being particularly high in broom sorghum grain and rice straw. The most extreme populace was recorded for broom sorghum grain and rice straw being 5.8 x $10^9$and 5.3 x 10^9 CFUs g-1, respectively so, broom sorghum grain could be recommended as suitable fermentation medium for the industrial-scale generation of *Trichoderma asperellum* OR234761strain. For *Trichoderma* formulation powder, it was possible to preserve conidial reasonability at room temperature 25ºC for 12 months at concentrations over 2.5 x 10^6 CFU/g. There were few aspects utilized by *Trichoderma asperellum* to empower plant development, these incorporate, indole acidic corrosive generation, hydrogen cyanide and ammonia generation as well as phosphate solubilization and siderophores production. *Trichoderma asperellum* OR234761 was tolerant strain because of their resilience capacity under abiotic stresses condition.

Keywords: *Trichoderma*, mass production, solid substrate fermentation, shelf life, PGPF, abiotic stress.

1. Introduction

Plant growth-promoting fungi (PGPF) are viewed as a potential alternative to chemical pesticides and fertilizers that have a negative impact on the environment (Harman *et al.,* 2004). *Trichoderma* spp. is a well-known PGPF that can successfully encourage plant development (Shoresh *et al.,* 2010). *Trichoderma* species in particular exhibit variety in encouraging plant growth since they can create phytohormones, break down organic matter, and shield plants from biotic and abiotic stressors (Cai *et al.,* 2015; Druzhinina *et al.,* 2018). Furthermore, *Trichoderma* species can enhance plant growth through a variety of processes, including the solubilization of insoluble phosphate and the synthesis of plant hormones like IAA and siderophore (Napitupulu *et al.,* 2019 and Reghmit *et al.,* 2022). However, *Trichoderma* spores must be applied in large quantities to soil for effective plant promotion (Zhang *et al.,* 2016).

Solid state fermentation (SSF) offers numerous advantages over liquid fermentation (Pandey, 1994). SSF has been regarded as an effective method for producing spores. (Roussos, 1987; Muñoz *et al.,* 1995 and Scheffer, 1997). SSF is the most common strategy for *Trichoderma* mass generation. Sorghum, corn, rye and millets grains are utilized as *Trichoderma* substrates mass production. These grains are dampened, sterilized and inoculated with *Trichoderma* spp and brooded for 10 to 15 days. *Trichoderma* produces dull green coloured spores covering the entire grain. The ultimate item can be utilized for soil and seed treatment. Solid state fermentation is utilized for commercial level generation; the most drawback of this prepares that it is time devouring and repetitive. (Srivastavaet *et al.,* 2016).

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SSF-produced fungal spores have a longer shelf life than liquid-state fermentation (Viccini *et al.,* 2007). Furthermore, SSF is closer to the natural habitat than liquid-state fermentation, and many species effectively manufacture spores by SSF (Singhania *et al.,* 2008). Solid materials such as sugarcane bagasse, wheat bran, sorghum mash, and coffee or tea squanders have been employed as substrates for *Trichoderma* SSF, but the poor spore yields prevent *Trichoderma* commercialization on a large scale (Rayhane *et al.,* 2019). As a result, it is basic to look for a more reasonable crude fabric for *Trichoderma* advancement and sporulation. As it were when inquire about discoveries are transmitted from the lab to the field do innovations gotten to be attainable. In spite of having a high potential for disease management, *Trichoderma* may not be utilized as a spore suspension within the field. Hence, *Trichoderma* cultures ought to be immobilized in specific carriers and fabricated as definitions for basic application, capacity, commercialization, and field utilize. *Trichoderma* ought to have the following characteristics to develop a successful *Trichoderma* formulation: high rhizosphere competence, high competitive saprophytic capacity, upgraded plant development, ease of mass duplication, wide range of activity, great and dependable control, environmental safety, compatibility with other bioagents, and tolerance to drying up, warm, oxidizing operators, and UV radiations (Jeyarajan and Nakkeeran, 2000).

Shelf life is the most imperative figure which decides the quality of *Trichoderma* bioformulation. *Trichoderma* formulations based on talc, peat, lignite, and kaolin have a shelf life of 3 to 4 months. After 120 days, the CFU count of *Trichoderma* in talc formulation was reduced to 50%. The shelf life of the *Trichoderma* harizianum (PDBC-Th10) formulation in Bangalore was extended by employing several components (chitin and glycerol) and warm shock towards the conclusion of the log phase of fermentation, which amplifies the shelf life of talc-based formulation up to one year (Sriram *et al.,* 2010 and Sriram *et al.,* 2011).

2. Materials and Methods

2.1. Place of Work

The present study was conducted in the Biofertilizers Production Unit of Agric. Microbiol. Res. Dept., soil, Water and Environ. Res. Inst. (SWERI), Agriculture Research Center (ARC), Giza, Egypt.

2.2. Molecular identification of the *Trichoderma* isolate:

Trichoderma isolated already from Egyptian clay rhizospheric soils of faba bean healthy plants. Genomic DNA isolation from *Trichoderma* isolate which grown on liquid potato dextrose agar medium (PDA), separated mycelia by filtration under aseptic condition and introduced to PCR studies. Amplification of the fungal ITS region was carried out with forward ITS1 (5′ TCC-GTA-GGT-GAA-CCT-GCG-G 3′) and reverse ITS4 (5′TCC-TCC-GCT-TAT-TGA-TAT-GC 3′) primers (White *et al.,* 1990). The polymerase chain reaction program was performed as previously described (Hewedy *et al.,* 2020). PCR studies were performed in ARC gene bank to sequence the sample's PCR product. The PCR sample product sent to Potsdam Institute of Biochemistry and Biology (Potsdam, Germany) used an ABI sequencer to do DNA sequencing. The sequence was submitted to the NCBI Gen-Bank in the United States to obtain an accession number. NCBI-GenBank assigned the accession number (OR234761 HM1) (Altschul *et al.,* 1990).

2.3. Organic Substrates

This study used five agricultural wastes or by product for grown identified *Trichoderma asperellum*. Rice straw, broom sorghum grain (*Sorghum vulgare* var. *technicum*), peanut pod, barley grain, and water fern (*Azolla filiculoides*) were among them.

2.4. Solid-Substrata Fermentation

All substrates were soaked in tap water overnight for *Trichoderma* biomass production, and surplus water was depleted. At that point, 300 g of each substrate was set in 1 L funnel shaped jars and sterilized three times in an autoclave at 121^0 C for 30 minutes over three days. After bringing to room temperature, they were inoculated under aseptic conditions with three 5 mm mycelia plates cut from the edge of 7 days culture of *Trichoderma asperellum* on PDA medium. The flasks were then stored at ambient laboratory temperature (25-30ºC) for 21days and shaken by hand on a regular basis. *Trichoderma* inocula were expelled from jars after brooding, air dried for up to 7 days in a clean room (Figure 1), and ground to a powder with mortar and pestle (Shahram Naeimi *et al.,* 2020).

Fig. 1: Schematic representation of the SSF framework for 21 days.

2.5. Determination of spore production on diverse organic substrates

Conidial generation of *Trichoderma asperellum* in all substrates was examined using serial dilution method on potato dextrose agar medium. 1 g dried test of each strong fermented substrate was suspended in 9 mL sterile distilled water prior to plating. Suspensions were rapidly agitated for 1 minute using a vortex mixer before serial dilutions and 200 µ L aliquots were put and speared over the surface of PDA medium in Petri dishes. The plates were brooded in the dull at 25°C for 3-5 days, until *Trichoderma* colonies were unmistakable. The colonies were counted and calculated as colony-forming units (CFUs) per gram of dry substrates.

2.6. Viability and Shelf Life

Five hundred grams of any dry tested substrates were weighted and placed in sterile plastic screw bags with three replicates for each substrate and stored at (25^0 C) and (4^0 C) until use. The shelf life of *Trichoderma asperellum* conidia was determined after one month, three, six, and twelve months. To count the shelf life samples each containing 1 g were taken from each dried substrate and suspended in 9 mL distilled water. The conidial generation and viability were evaluated as already depicted. The formulations' capacity of shelf life was evaluated as colony-forming units (CFUs) per gram of dry substrates.

2.7. Screening for PGPF activity of *Trichoderma asperellum*:

2.7.1. Qualitative hydrogen cyanide (HCN) estimation

Screened HCN qualitatively production. *Trichoderma asperellum* isolate was inoculated on Potato Dextrose Agar (PDA) medium supplemented with 4.4 g/L glycine. Impregnated filter paper with alkaline picric acid solution was fixed on the bottom of the plate cover. The plates were incubated for 7 days at 26 ± 2^0 C. The creation of HCN was noticed by changing in the color of the filter paper from

yellow to brown or reddish brown according to the procedures described by Bakker and Schipper (1987).

2.7.2. Phosphate solubilization

Screened method to determined phosphate solubilization using the solid medium contained tri calcium phosphate as insoluble inorganic phosphorus source (Pikovskaya agar). Each plate was inoculated with 6mm agar disc of 5 day-old fungal culture *Trichoderma asperellum* into medium and incubated at 26 ± 2^0 C (Gupta *et al.,* 1994). The mineral phosphate dissolved obtained by the presence of a clear zone around the fungal colony (Noori and Saud 2012). The solubilization index (IS) was calculated by the ratio of the total diameter (halo + colony) and the colony diameter (Edi-Premono *et al.,* 1996).

2.7.3. Qualitative production of ammonia (NH_3)

Broth culture peptone water was screened microbial ammonia production of *Trichoderma asperellum* by adding 1 ml of Nessler's reagent to each tube after incubation at 25ºC for72h. The positive result for ammonia production was recorded by color development from yellow to brownish orange (Bakker and Schipper 1987).

2.7.4. Production of siderophores

Evaluate the method to siderophores production by *Trichoderma asperellum* on malt extract agar medium containing 8-hydroxyquinoline (50 mg L^{-1}). The positive siderophores production result was recorded by tested strains growth on this media after 5 days of incubation at 26 ± 2°C according to Hoyos-Carvajal *et al.* (2009).

2.7.5. Indole acetic acid (IAA)

Trichoderma asperellum isolate was screened for its ability to produce Indole Acetic Acid (IAA) using potato dextrose broth medium (PDB) which supplemented with L-tryptophan as precursor according to the method of Bric *et al.* (1991). The inoculated were putted on rotary shaker (150 rpm at 28°C) for 3 days, then the cultures were centrifuged (1500 rpm for 3 min.) and 2 ml of Salkowski reagent were added to each 2 ml of supernatant and measured at wave length 530 nm for qualitatively. The red color revealed IAA production.

2.8. *Trichoderma* isolate and abiotic stress tolerance:

2.8.1. High temperature tolerance:

Trichoderma asperellum isolate was inoculated on potato dextrose plates medium and incubated at 30°C, 45°C and 50°C for 7 days. Noticed the growth was compared to the growth in the control plates which were incubated at 30°C.

2.8.2. Salinity tolerance:

Trichoderma asperellum isolate was inoculated on PDA medium which upplemented with 1%, 2% and 3% NaCl and incubated for 7 days at 30°C in comparison with control without NaCl.

2.8.3. Drought tolerance

1mm disc of *Trichoderma asperellum isolate* was transferred to the PDA broth medium containing 10%, 20%, 30 and 35% of polyethylene glycol (PEG 6000 Da) and cultured for 7 days at 30°C. As previously mentioned before, the percentage reduction in growth in PEG modified medium was computed (Leo Daniel *et al.,* 2011).

3. Results and Discussion

3.1. Molecular identification of the *Trichoderma* strain:

Based on the discoveries of DNA sequencing performed by the Potsdam Institute of Biochemistry and Biology (Potsdam, Germany) with an ABI sequencer. The sequence was submitted to the NCBI Gen-Bank in the United States to obtain an accession number. NCBI-GenBank assigned the accession number (OR234761). The normal length of the sequence was 544 bp, and the BLASTN accommodation

comes about appeared that the species *Trichoderma asperellum* had a high similarity and identity of over 99%. *Trichoderma asperellum* was recognized as the sample species by the results.

3.2. Colonization and Sporulation

Trichoderma asperellum OR234761 colonized all substrates at varying degrees of intensity after one month, ranging from sparse development to covering the whole substrate (Table 1). The solid medium at a first had a white appearance due to mycelium arrangement, but the color gradually changed and diverse shades of green were recognized among the substrates. Besides, the level of conidia varied enormously among the substrates, and the results in Table 1 demonstrated that the influence of different substrates on the fungal population after one month of incubation was significantly differed with $5.8x10^9$ and $5.3x10^9$ CFUs g-1, respectively, broom sorghum and rice straw grain had the maximum conidia formed. However, the two mentioned before substrates, peanut pod and barley grain were not significantly different with $3.3x10^7$ and $3.1x10^7$ CFUs g-1 respectively. Water fern (Azolla) was discarded due to inadequate colonization ($1.3x10^5$ CFUs g-1).

Table 1: Colonization of *Trichoderma asperellum* OR234761 in numerous substrates and its starting and last populations.

Raw materials	Colonization	Count after 1 month at 25°C	Count after 3 month at 25°C	Count after 6 month at 25°C	Count after 12 month at 25°C	Count after 1 year at 4°C
Broom sorghum	+++High	$5.8x10^9$	$2.1x10^9$	$2.6x10^7$	$2.5x10^6$	$2.5x10^4$
Rice straw	+++ High	$5.3 x10^9$	$3.1x10^8$	$2.1x10^6$	$1.1x10^5$	$2.1x10^4$
Peanut pod	++Medium	$3.3x10^7$	$3.3x10^7$	$2.7x10^5$	$3.3x10^4$	$1.8x10^3$
Barely grain	++Medium	$3.1x10^7$	$2.8x10^7$	$2.5x10^5$	$3.3x10^4$	$1.3x10^2$
Water fern	+Low	$1.3x10^5$	$3.4x10^4$	$2.4x10^3$	$2.2x10^2$	$1.1x10^2$

3.3. Viability and Shelf Life

The shelf life of *Trichoderma asperellum* OR234761 designed product is critical to effective marketing. The population of *Trichoderma asperellum* OR234761 on the substrates remained practically stable or fell very slowly after one month of incubation at room temperature (Table 1), in many case the number of CFUs diminished with time in all five substrates during the 12-month capacity period at 25 °C. The population pattern in broom sorghum grain and rice straw, which come to greatest starting populations after one month ($5.8 x 10^9$ and $5.3 x 10^9$ CFUs g^{-1}) respectively, gradually fell until Month three ($2.1x10^9$ and $3.1x10^8$ CFUs g-) after 6 months, the population in both substrates mentioned before was dropped sharply to $2.6 x 10^7$ and $2.1 x 10^6$ CFUs g^{-1}, respectively., and by 12 months, it was dropped to $2.5 x 10^6$ and $1.1 x 10^5$ CFUs g^{-1}, respectively. The fungal population in peanut pod and barely grain stored at room temperature was similar until storing period reached 6 months followed by dramatically decease when determined after 12 months being$3.3x10^4$ and $3.3x10^4$ CFUs g^{-1}, respectively. The population of *Trichoderma asperellum* on the water fern was reduced beginning in the first month of storing and gradually decreased reached $2.2x10^2$ CFUs g^{-1} after 12 months of storing at room temperature. In comparison, the populations of *Trichoderma asperellum* OR234761 in all substrates steadily declined after a year of storage at 4°C.

In fact, *Trichoderma* members are saprophytic fungi that grow abundantly on a variety of organic substrates in nature (Kredics *et al.,* 2014). Singh *et al.* (2007) reported the impact of different agricultural wastes on the conidiation and shelf life of a *T. asperellum* strain; in any case, utilized tea leaves and wheat bran-sawdust which effectively affected population number and shelf life, individually. The current study found that *T. asperellum* AS12-2 thrived and sporulated on all five solid substrates tested, however the extent of colonization and biomass production varied depending on the growth medium. This distinction reflects the diverse components in organic substrates as well as the *Trichoderma* strain's feeding preferences. The physicochemical properties of organic substrates have a significant impact on the fermentation process (Li *et al.,* 2019). Rice-based substrates are the foremost regularly utilized media for the development and sporulation of numerous fungi (Sala *et al.,* 2019). Rice straw is high in cellulose, hemicellulose, and lignin, making it an excellent source of nutrients for *Trichoderma* strains' rapid development and sporulation (El-Tayeb *et al.,* 2012). After one year of storage at room temperature, broom sorghum grain and rice straw had the highest population and were chosen as the most excellent substrates with the longest shelf life. *T. asperellum* OR234761 vitality

decreased in all 5 substrates during refrigerator incubation, although the population drop was few compared to that at room temperature, and the ultimate populace was quite robust. Fungal essentialness most elevated in broom sorghum grain and rice straw, as anticipated, when compared to the other substrates.

3.4. Screening of *Trichoderma asperellum* OR234761 for PGPF activity:

Trichoderma species are well known as bio-fertilizer fungi due to their ability to create phytohormones and promote plant development substances (Table 2). *Trichoderma* spp. has long been utilized in agriculture as a plant growth promoter and as a biopesticide to combat soil-borne diseases. Plant growth promoting properties (PGP) of *Trichoderma asperellum* OR234761was illustrated in this consider. *Trichoderma* tested was found productive for the generation of phytohormones and biomolecule that actuate plant development. *Trichoderma asperellum* OR234761was tested for its ability to create phytohormones and metabolites that stimulate plant development in our current investigation.

The qualitative screening of HCN production demonstrated that *Trichoderma asperellum* OR234761 could create HCN. The color of the filter paper extended from yellow to ruddy brown, showing that the amount of HCN created extended from minor to critical.

According to the results of the qualitative evaluation of phosphate solubilization, *Trichoderma asperellum* OR234761 was able to solubilize insoluble inorganic phosphates on medium Pikovskaya's, achieving a noticeable halo-zone of 65mm (Table 2). As well as a solubilization index (SI) of 1.85. Data also showed that IAA synthesis by *Trichoderma asperellum* OR234761. IAA could be produced and its amount was 25.55 µg/ ml.

Table 2: Screening of Trichoderma asperellum OR234761for PGPF activity, IAA production, Phosphate solubilization, solubilization index (SI), HCN production, Ammonia production and Siderophores production.

PGPF activity	*Trichoderma asperellum* OR234761
IAA production	25.55 µg/ ml
Phosphate solubilization	65mm
solubilization index (SI)	1.85
HCN production	++
Ammonia production	++
Siderophores production	13.22%

Based on color fluctuation, the *Trichoderma asperellum* OR234761 was capable of producing ammonia and siderophores. Lalngaihawmi and Bhattacharyya (2019) who detailed that the sum of IAA created by *Trichoderma* spp was extending from 6.32 µg mL^{-1} to 13.38 µg mL^{-1}. Besides, in previous considers, *Trichoderma asperellum* strain CHF 78 shows different plant growth-promoting properties, including phosphate-solubilizing capacity and siderophores synthesis (Li *et al.,* 2018). Furthermore, our findings demonstrated that *Trichoderma* isolates may deliver HCN, which bolsters the discoveries of earlier investigate that detailed positive HCN generation by *Trichoderma* spp. (Mohiddin *et al.,* 2017). Importantly, the potential of *Trichoderma* isolates to produce ammonia was demonstrated. Our conclusion is also supported by, Ahemad and Kibret (2014) who expressed that ammonia is advantageous to plants, either directly or indirectly. Ammonia generation by *Trichoderma* isolates may have an indirect impact on plant growth; ACC created in plant tissues by ACC synthase is discharged from plant roots and taken up by adjacent microorganisms. *Trichodrema* may at that point hydrolyze ACC (1-aminocyclopropane-1-carboxylic acid) to create ammonia. Besides, the result of the generation of ammonia is maintained with results by Mohiddin *et al.* (2017) who detailed that among 20 *Trichoderma* spp. isolated from chilli rhizosphere, 13 isolates were able to create ammonia.

3.5. Abiotic stress tolerance of *Trichoderma asperellum* OR234761.

3.5.1. High temperature

The test strain *Trichoderma asperellum* OR234761 illustrated viable resistance. At 30°C, $3.2x10^8$ cfu/g were found, whereas at 45°C, cfu were $1.8x10^7$/g and encourage diminished at 50°C ($1.1x10^7$ cfu/g).

3.5.2. In the occurrence of salinity

Obtained results reflected significant dropped in development as the salt concentration within the medium increased. When compared to the control plate, addition 1% salt to media showed 92.9% growth, followed by 2% (85.6%), 3% (69.4%), 4% (35.2%), 5% (21.1%) growth (Fig. 2).

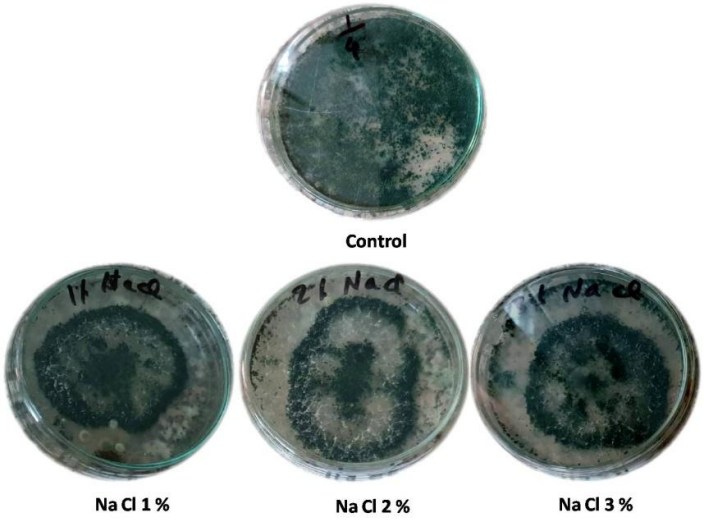

Fig. 2: Salinity stress tolerance of *Trichoderma asperllum* OR234761.

3.5.3. Drought tolerance

Data illustrated in Figure (3) exhibited gradually slight deficiency in growth with increasing PEG concentration. The growth was ranged from 78.3% at 10% PEG to 63% in 35% PEG.

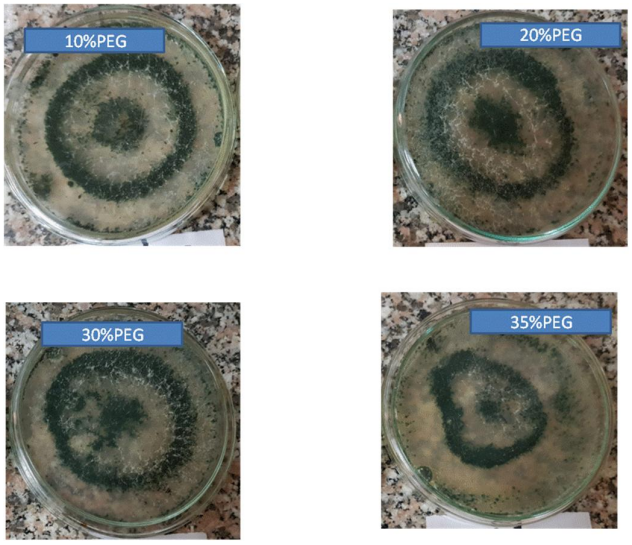

Fig. 3: Drought stress tolerance of *Trichoderma asperllum* OR234761.

Widden and Hsu (1987) discovered that the capacity of distinctive *Trichoderma* species to colonize pine or maple litter changed with temperature. The current strain was assessed for abiotic stress resistance since stress tolerant strains can be productively deployed in serious circumstances where they can illustrate greater rhizosphere competence and saprophytic competitive capacities. Surprisingly, a few of the abiotic stress resistant microorganisms also secured plants from abiotic pressures such as drought (Timmusk and Wagner (1999), salinity (Han and Lee 2005), a terrifying harm (Ait Barka *et al.,*

2006), and high temperature (Ali *et al.,* 2009). A few recent studies have shown that these fungi reduce abiotic stressors. According to field research, they may impart drought resistance, at least in part, by promoting deeper root penetration into the soil profile (Harman, 2000). According to a later study, *T. hamatum* boosted cocoa plant resilience to water shortfall by boosting root development, which advertised more water resources to treated plants and postponed the starting of water shortage in these plants (Bae *et al.,* 2009). Drought stress expanded proline and soluble protein aggregation, which was boosted assist by *T. harzianum* inoculation. Plants with higher levels of free proline show more stress resistance (Abd_Allah *et al.,* 2015). Proline has an imperative part in ROS scavenging and protein and membrane structure maintenance (Ahanger *et al.,* 2014). Moreover, it progresses energy generation and capacity by controling nitrogen metabolism (Ahanger *et al.,* 2015; Hashem *et al.,* 2015). Proline preserves metabolic processes amid stressful conditions by replacing water, giving stability to critical cellular structures. (Zhifang and Loescher 2003). *T. harzianum* boosted phenol synthesis in plants, which advanced development in drought-stressed tomato plants and secured them from oxidative stress by expelling ROS. Plants advantage from expanded phenol substances by working as free radical scavengers and intervening cell wall development (Alwhibi *et al.,* 2017). *Trichoderma* produces a number of chemicals that stimulate resistance to biotic and abiotic stressors (Harman *et al.,* 2004).

4. Conclusion

It can be concluded from the display consider that broom sorghum grain may be suggested as reasonable fermentation medium for the industrial-scale production of *Trichoderma asperellum* OR234761strain. *Trichoderma asperellum* OR234761 is a cosmopolitan fungal organism that has been utilized not only in enhancing plant growth but also abiotic stress tolerant.

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