Screening of Organic Substrates for Solid-State Fermentation, Viability and Bioefficacy of *Trichoderma harzianum* AS12-2, a Biocontrol Strain Against Rice Sheath Blight Disease

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Abstract: The present study was undertaken to find the most suitable organic substrates for the biomass production, viability and efficacy of the biocontrol strain *Trichoderma harzianum* AS12-2 in the solid-state fermentation system. In total, 13 inexpensive, locally available substrates (agricultural wastes or by-products) were inoculated with the antagonist, and following one month of incubation at room temperature, all colonized substrates were air dried and ground to powder. The shelf life and viability of the *Trichoderma* strain were assessed as colony-forming units per gram (CFUs g^{-1}) of each substrate on a monthly basis for up to one year at room temperature (25 ± 2 °C) and in the refrigerator (4 °C). In order to find out the effect of the substrate on the bioefficacy of *T. harzianum* AS12-2, the biocontrol potential of the formulations was evaluated against rice sheath blight disease caused by *Rhizoctonia solani*. The results showed that the fungus colonized more or less all substrates after one month, although the degree of colonization and conidiation was different among the substrates, being especially high in broom sorghum grain, rice husk, rice straw, rice bran and sugar beet pulp. Analysis of variance (ANOVA) of the population in the substrates in "Month 0" showed that the effect of treatment was significant, and the means were significantly different. The maximum population was recorded for broom sorghum grain and rice straw (6.4 × 10^{10} and 5.3 × 10^{10} CFUs g^{-1}, respectively). The population declined in all substrates after one year of incubation at room temperature. This decline was relatively smaller in broom sorghum grain, rice straw and rice husk. On the other hand, the population in the same substrate incubated in the refrigerator was decreased in a mild slope, and the final population was high. In addition, the results of greenhouse assay showed that all bioformulations were effective in controlling the disease, and there were no significant differences among the substrates. According to the results of this study, broom sorghum grain, rice husk, rice straw, sugar beet pulp and cow dung could be recommended as suitable fermentation media for the industrial-scale production of *T. harzianum* strains.

1. Introduction

The application of chemical pesticides is the most common way to protect plants against pests and diseases in agriculture. However, the use of synthetic compounds adversely affects the environment and human health [1,2]. In addition, the high cost of investigating, developing and registering new synthetic pesticides, and the rapid emergence of pathogen resistance, have also contributed to an increased interest in finding alternate disease control methods [3]. In recent years, the global production, registration and application of biological pesticides in agriculture as an alternative to chemicals has been rapidly increasing due to public concerns about human health, food safety, and impact on the environment [4,5]. Many companies are attracted by the idea of developing environment-friendly products, and several commercial biopesticides are currently being used for the efficient control of plant disease, resulting in the improved productivity of many crops [6]. Biocontrol based on antagonistic microorganisms is an effective and sustainable approach that has numerous advantages, including increased crop yield, safety for humans and other non-target organisms, reduction of pesticide residues in food, water, and soil, as well as increased biodiversity [7–9].

Once biocontrol agents (BCAs) are identified and proven to be effective against plant pathogens, they have the potential to be developed into commercial biopesticides [10]. However, the development of a microbial product is a time-consuming and costly procedure, and in order to commercialize a beneficial microbe, there are some obstacles to its production, formulation, registration and field application [6]. The formulation of BCAs and their bioefficacy greatly depends on the quantity and quality of fungal biomass [11,12]. Nevertheless, a crucial aspect in the commercialization of a microbial biopesticide is the accessibility of an economical mass production technology that provides an abundant and effective form of the biocontrol agent [13,14]. A fermentation substrate should be nutrient-rich, low-cost and easily available, resulting in the production of sufficient biomass, containing potent inoculum [15,16]. Moreover, the biomass of fungal biocontrol agents must survive several processing steps, including harvesting, drying, formulation, storage and delivery [17]. In this sense, poor shelf life is regarded as a major obstacle to commercializing microbial biopesticides [18]. The shelf life of the microbial product should be improved by using appropriate organic substrates via a suitable production method that results in biomass of both high quantity and quality [19].

Solid-state fermentation (SSF) and liquid state fermentation or submerged fermentation (SmF) are two methods for the propagule production of fungal biocontrol agents [15]. SSF has appeared as a suitable technology for the production of microbial biomass [20], and is defined as a process that involves the cultivation of microorganisms on an organic solid substrate, which is generally a non-soluble material that serves as physical support as well as nutrient source [21]. Filamentous fungi are the most frequently used microorganisms for SSF technology [22]. SSF is applied at the commercial scale in the industry for the production of value-added products, such as enzymes, secondary metabolites, antibiotics, organic acids, biofuels, conidia and biomass, by a variety of fungi [23,24]. It is performed in various containers, such as plates, beakers, flasks, bottles, bags, trays and column bioreactors [25]. SSF has noticeable economic potential in the biopesticide industry [26,27], and offers several advantages over SmF, including higher fermentation productivity, higher product stability, lower capital and operating costs, simpler equipment and media, lower water and energy requirements, less technical difficulties, no need to control several parameters, easier downstream processing and lower demand on sterility due to the low water activity [27–31]. SSF simulates the natural conditions and habitat for fungi [22,32], and it is well adapted to the metabolism of these microorganisms [20]. The fungal spore is the most resistant propagule that survives adverse environmental conditions, especially desiccation [29,33]. SSF contributes to a higher number of hardy and healthy spores with longer viability and higher resistance to UV and other abiotic factors [34–37]. Moreover, SSF is suitable for producing fungi that do not sporulate in liquid media, or do not tolerate the SmF conditions [38]. Generally, organic substrates in SSF act as carriers for the biomass, and it is not necessary to develop a complicated formulation afterward [38]. Thus, SSF may be appropriate in developing countries where agricultural wastes are available, high-tech equipment is limited, and the workforce is abundant [39].

Another advantage of SSF over SmF is that SSF allows the valorization of agro-industrial wastes or by-products, so it provides a sustainable recovery of the resources [22,31,40]. However, SSF has some disadvantages, including higher fermentation time, difficulties on scale-up and in control of process parameters (i.e., substrate moisture level, pH, temperature), as well as problems with heat build-up. Furthermore, homogenizing the solid substrate is not possible, causing temperature and composition gradients [25].

Production costs, partly due to the expensive substrates and low biomass yields, are among the most important reasons for the limited number of biofungicides on the market [41,42]. However, in the SSF process, the microorganism is cultured on different types of organic matter, including agricultural wastes and by-products which are available at low cost. Various substrates can serve as suitable media for the production of inoculum of biocontrol fungi, including cereal grains (wheat, barley, corn, millets and sorghum), cereal brans, cereal straws, husks, meals, plant-based oil cakes, bagasse, molasses and sawdust [22,24,29,43,44].

Trichoderma species are versatile, ubiquitous and abundant fungi found in many soil types in diverse ecosystems [45]. They have been known as biocontrol agents of plant pathogens for decades, and collectively are effective against various diseases of many crops in the greenhouse, nursery, field and orchards [46,47]. *Trichoderma* strains can act on plant pathogens through a number of mechanisms, such as antibiosis, hyperparasitism, enzyme production, induction of plant resistance, and competition for essential nutrients and space [48–50]. Additionally, these microbial agents also promote plant growth and abiotic stress tolerance against such disruptions as drought and salinity [51–54]. Members of the genus *Trichoderma* are widely studied fungi, and are among the most commonly used microbial BCAs in agriculture [55]. Owing to the multiple beneficial effects on plants, *Trichoderma* spp. comprise the majority of commercial fungal BCAs in agriculture, and are presently marketed as biopesticides, biofertilizers, growth enhancers, and stimulants of natural resistance [8,56,57]. The number of *Trichoderma*-containing products found on the international market has been rapidly growing to more than 250 available products [55].

Considerable amounts of studies have focused on the mass production of *Trichoderma* spp. in the hope of developing effective and safe bioproducts as substitutes for chemicals for the control of plant diseases [17,58–61]. The most common formulations of *Trichoderma* are wettable powder and granules [55,62], and SSF is a routine biological system for mass production [20,23,25,42,44,63–67].

The conidia of *Trichoderma* are the most efficient propagules that survive downstream processing [68]. For instance, the conidia of *T. harzianum* produced in aerial mycelium with SSF persist longer under adverse environmental conditions than those produced by SmF, and the wall thickness of aerial conidia is almost twice that of submerged ones [69]. Nevertheless, *Trichoderma* strains produce high levels of biomass, as well as conidia and chlamydospores in SSF [70]. Various agricultural waste materials, such as cereal grains, straw and brans, as well as sugarcane bagasse, ground corn cobs, sawdust, etc., have been frequently used to produce *Trichoderma* biocontrol strains [10].

The objective of the present study was to find, amongst 13 inexpensive and locally available substrates (agricultural wastes or by-products), the most suitable organic substrate for the colonization, conidiation, viability and bioefficacy of a biocontrol strain of *T. harzianum* against rice sheath blight, and to develop a low-cost solid-state fermentation process that could be used at the industrial scale.

2. Materials and Methods

2.1. Biocontrol Strain

Trichoderma harzianum AS12-2, a highly effective strain against *Rhizoctonia solani* in vitro and the rice sheath blight disease caused by this pathogen under greenhouse and field conditions [71–73], was used in this study. It was originally isolated from paddy soil and identified during previous studies on the basis of morphology as well as the sequence analysis of rDNA-ITS [71] and a fragment of the *tef1α* gene [73] (GenBank accession numbers: EU821789 and FJ618586, respectively).

2.2. Organic Substrates

Thirteen locally available and low-cost organic substrates, which are considered as agricultural wastes or by-products, were involved in this study. They were rice husk, crushed (powdered) rice husk, rice straw, rice bran, wheat bran, broom sorghum (*Sorghum vulgare* var. *technicum*) grain, sawdust, cow dung, sugar beet pulp, soybean meal, peanut pod, used barley grain (obtained from a local insect mass rearing unit), and water fern (*Azolla filiculoides*).

2.3. Solid-State Fermentation

For biomass production of *T. harzianum* AS12-2 [71–73], all substrates were soaked in tap water overnight, and excess water was drained out. Then, 300 g of each substrate was filled in 1 L conical flasks and sterilized in an autoclave three times at 121 °C for 30 min during three successive days. After cooling the substrates to room temperature, they were inoculated under aseptic conditions with three 5 mm mycelial discs cut from the edge of 2-day-old PDA cultures of the biocontrol fungus in order to have a uniform and full colonization of the organic substrates, then the flasks were sealed with sterile cotton wool and kept at ambient laboratory temperature for 30 days and periodically shaken by hand in order to have uniform growth of the BCA (Figure 1A). After incubation, *Trichoderma* inocula were taken from the flasks, air dried for up to 7 days in a clean room (Figure 1B), and ground to a powder with Mortar Grinder PULVERISETTE 2 (Fritsch, Germany).

Figure 1. Solid state fermentation of *T. harzianum* AS12-2 strain on broom sorghum grains. (**A**) Fermentation flask. (**B**) Grains colonized by *T. harzianum* after drying.

2.4. Sporulation

Conidium production of *T. harzianum* AS12-2 in all substrates was examined by the serial dilution method on potato dextrose agar (PDA, Merck, Germany) medium. Prior to plating, 1 g dried sample of each solid fermented substrate was suspended in 9 mL sterile distilled water. Suspensions were vigorously shaken using a vortex mixer for 1 min, then serial dilutions were made and 200 μL aliquots were plated on PDA medium in Petri dishes. The plates were incubated for 3–5 days in the dark at 25 °C, until *Trichoderma* colonies were visible. The number of colonies was enumerated visually, and results were recorded as colony-forming units (CFUs) per gram of dry substrates.

2.5. Viability and Shelf Life

All dried substrates were packed into 50 mL sterile plastic screw cap bottles (Falcon Plastics, Brookings, SD, USA) with three replicates for each temperature, sealed and stored at room temperature (25 ± 2 °C) and in the refrigerator (4 °C) until use. The shelf life of conidia of *T. harzianum* AS12-2 was assessed after 1 month and then monitored at a 3-month interval for 12 months. To determine the shelf life of conidia, 1 g samples from each dried substrate kept at each temperature were taken from

the container, suspended in 9 mL sterile distilled water, and conidial production and viability were estimated as described above. The shelf life of the formulations during storage was expressed as log CFU g^{-1}.

2.6. Biocontrol Efficacy

An experiment was conducted to determine the biocontrol efficacy of the five best formulations (according to the results of colonization and shelf life studies) in relation to the rice sheath blight disease in greenhouse, according to Naeimi et al. [71]. The rice plants (cv. Shiroodi) were inoculated with *R. solani* RBL1 (obtained from the culture collection of the Rice Research Institute of Iran). A 5-mm mycelial plug of the pathogen was placed between the junction of the basal leaf sheath and the stem above the water line at the maximum tillering stage. Three rice plants (five tillers per hill) were inoculated for each treatment. Biocontrol suspensions were made from each colonized substrate and the concentration of spores was adjusted to 10^7 conidia per ml using a Neubauer Improved haemocytometer (Precicolor, HBG, Giessen, Germany). For each replicate plant, 50 mL of biocontrol suspension was evenly sprayed on the rice phyllosphere using a hand sprayer at two stages: 24 h after inoculation of the pathogen and seven days after the first spray. Propiconazole (TILT®, 250 EC, Syngenta, Switzerland) was used as the chemical fungicide for comparison and applied as a foliar spray (1 L ha^{-1}). Plants inoculated only with *R. solani* were considered as infected control, while plants sprayed with distilled water served as non-infected control. Disease severity was recorded following the International Rice Research Institute's standard evaluation procedure for rice [74] as relative lesion height (RLH, the average vertical height of the uppermost lesion on leaf or sheath expressed as a percentage of the average plant height) 28 days after inoculation of the pathogen using the formula [71]:

$$\text{RLH (\%)} = 100 \times [\text{Highest point a lesion occurred (cm)/Plant height (cm)}].$$

2.7. Statistical Analysis

All experiments were established as a completely randomized design with three replicates. The data were analyzed for significance with analysis of variance (ANOVA) using the SAS software ver. 9.1 (SAS Proc GLM; SAS Institute, Inc., Cary, NC, USA). The CFU values were transformed to log$_{10}$ before statistical analysis. All means of the treatments were compared by Duncan's multiple range tests at $p \leq 0.01$.

3. Results

3.1. Colonization and Sporulation

The results showed that after one month, *T. harzianum* AS12-2 colonized all substrates to different intensities, from scanty growth to covering the whole substrate (Table 1). The solid media had a white color at first due to the development of the mycelium, then the color gradually changed and various shades of green were observed among the substrates. In addition, the level of conidiation was significantly different among the substrates, and the results of ANOVA showed that the effect of treatment on the fungal population in "Month 0" (after one month of incubation, air drying and grinding of the substrates) was significant at $p \leq 0.01$. The highest conidiation occurred in broom sorghum grain and rice straw with 6.4×10^{10} and 5.3×10^{10} CFUs g^{-1}, respectively, which were placed into one single statistical group (Table 1). Next to these two substrates, rice bran, rice husk and sugar beet pulp represented the highest populations, with 4.7×10^9, 3.1×10^9 and 2.3×10^9 CFUs g^{-1}, respectively, which were not significantly different (Table 1). Soybean meal showed the least fungal population with scanty colonization and white fungal mass. Soybean meal, together with waste barley grain, water fern and sawdust, were abandoned due to poor colonization and conidiation of the biocontrol fungus, and the nine remaining substrates were selected for further studies.

3.2. Viability and Shelf Life

After one month of incubation at room temperature, the population of *T. harzianum* AS12-2 on the substrates remained almost stable or decreased very slowly (Figure 2A), however in all nine substrates, the number of CFUs reduced with time during the 12 months of storage. The pattern of population decline in broom sorghum grain and rice straw, in which the maximum initial populations were observed (6.4×10^{10} and 5.3×10^{10} CFUs g^{-1}, respectively), was similar. The population of the biocontrol fungus in both substrates declined slowly until Month 3, and then sharply decreased, and at the end reached 2.5×10^9 and 1.1×10^9 CFUs g^{-1}, respectively, in Month 12. In rice husk and sugar beet meal, the population dynamics of the fungus was similar until Month 6. In fact, the population rose slowly until Month 3 and then dropped sharply until Month 6. The population decline continued to Month 12 with a larger decrease in sugar beet meal, and the final populations were counted as 3.3×10^7 and 3.3×10^6 CFUs g^{-1}, respectively. The reduction of the population in the crushed rice husk started from Month 1 and continued slowly to Month 12, reaching 2.2×10^7 CFUs g^{-1}. The population changes in rice bran and wheat bran were similar: the viability of the biocontrol fungus rapidly reduced in both substrates from Month 2 to Month 12, but this reduction was considerably higher in wheat bran. The CFU numbers of the fungus per grams of rice and wheat bran were 1.2×10^7 and 5.8×10^5, respectively, at the end of Month 12. The populations of *T. harzianum* AS12-2 in peanut pod and cow dung declined similarly during the studied period, but showed a considerable decrease in peanut pod in Month 12, and finally the populations were calculated as 6.6×10^6 and 1.5×10^7 CFUs g^{-1}, respectively.

Table 1. Colonization of *Trichoderma harzianum* AS12-2 in different substrates and its initial and final populations.

Substrate	Colonization [a]	Initial Population [b] (CFU g^{-1} Substrate)	Final Population [c] (CFU g^{-1} Substrate)	Final Population [d] (CFU g^{-1} Substrate)
Rice husk	+ + +	$3.1 \times 10^9 \pm 7.0 \times 10^{7\ bc}$	$3.3 \times 10^7 \pm 7.8 \times 10^{6\ b}$	$3.8 \times 10^8 \pm 2.2 \times 10^{7\ b}$
Crushed rice husk	+ + +	$1.2 \times 10^9 \pm 7.2 \times 10^{7\ cd}$	$2.2 \times 10^7 \pm 4.3 \times 10^{6\ bc}$	$1.6 \times 10^8 \pm 2.8 \times 10^{7\ c}$
Rice straw	+ + +	$5.3 \times 10^{10} \pm 4.7 \times 10^{9\ a}$	$1.1 \times 10^9 \pm 1.5 \times 10^{8\ a}$	$1.0 \times 10^{10} \pm 7.2 \times 10^{8\ a}$
Rice bran	+ +	$4.7 \times 10^9 \pm 5.8 \times 10^{8\ b}$	$1.2 \times 10^7 \pm 1.4 \times 10^{6\ cd}$	$7.8 \times 10^8 \pm 5.9 \times 10^{7\ b}$
Wheat bran	+ +	$7.0 \times 10^8 \pm 1.5 \times 10^{8\ de}$	$5.8 \times 10^5 \pm 8.9 \times 10^{4\ f}$	$1.1 \times 10^8 \pm 1.8 \times 10^{7\ c}$
Broom sorghum grain	+ + +	$6.4 \times 10^{10} \pm 5.2 \times 10^{9\ a}$	$2.5 \times 10^9 \pm 4.4 \times 10^{8\ a}$	$1.5 \times 10^{10} \pm 2.3 \times 10^{9\ a}$
Waste barley grain	+ +	$4.3 \times 10^7 \pm 1.2 \times 10^{7\ f}$	n/a	n/a
Peanut pod	+ +	$3.7 \times 10^8 \pm 9.6 \times 10^{7\ e}$	$6.6 \times 10^6 \pm 7.1 \times 10^{5\ de}$	$3.3 \times 10^7 \pm 9.5 \times 10^{6\ d}$
Sugar beet pulp	+ + +	$2.3 \times 10^9 \pm 6.9 \times 10^{8\ bc}$	$3.3 \times 10^6 \pm 7.8 \times 10^{5\ e}$	$6.5 \times 10^8 \pm 7.1 \times 10^{7\ b}$
Soybean meal	+	$1.4 \times 10^3 \pm 2.3 \times 10^{2\ h}$	n/a	n/a
Cow dung	+ + +	$2.7 \times 10^8 \pm 2.5 \times 10^{7\ e}$	$1.5 \times 10^7 \pm 2.3 \times 10^{6\ bcd}$	$9.0 \times 10^7 \pm 4.7 \times 10^{6\ c}$
Water fern	+ + +	$2.0 \times 10^6 \pm 3.8 \times 10^{5\ g}$	n/a	n/a
Sawdust	+	$3.2 \times 10^6 \pm 3.3 \times 10^{5\ g}$	n/a	n/a

(*a*) Colonization intensity, + + +: high; + +: medium; +: low. (*b*) Initial population of biocontrol fungus after one month of incubation of the substrates at room temperature (25 ± 2 °C). (*c*) Final population of the biocontrol fungus after 12 months of incubation of the substrates at room temperature (25 ± 2 °C). (*d*) Final population of the biocontrol fungus after 12 months of incubation of the substrates inside a refrigerator (4 °C). Data present mean values ± standard error from three replicates. Values in each column followed by the same letter in superscript are not significantly ($p \leq 0.01$) different according to the Duncan's multiple range test. n/a: not available (these substrates not included in viability analysis).

In comparison, the populations of *T. harzianum* AS12-2 in all substrates (except for rice husk and peanut pod) decreased gradually when they were refrigerated at 4 °C for over one year of storage (Figure 2B). In the rice husk (both crushed and uncrushed) and peanut pod substrates, the viability slowly declined until Month 3 and Month 6, respectively, then it sharply decreased afterwards. Likewise, the population gradually decreased in peanut pod until Month 6, and then it steeply declined. Despite the loss of viability of the biocontrol fungus in all substrates during their incubation in the refrigerator, this population decline was considerably low compared to the incubation of the same substrates at room temperature.

The initial and final populations of *T. harzianum* AS12-2 in all substrates at both temperatures are shown in Table 1. The results of ANOVA show that the effect of treatment on the fungal population in Month 12 (end of incubation) was significant at both temperatures ($p \leq 0.01$).

Table 2. Efficacy of different organic substrates containing *Trichoderma harzianum* AS12-2 in controlling rice sheath blight in the greenhouse.

Treatment	Disease Severity (%) [a]
Crushed rice husk	31.84 ± 3.14 [b]
Rice straw	30.24 ± 2.81 [b]
Broom sorghum grain	30.29 ± 2.18 [b]
Sugar beet pulp	34.94 ± 3.72 [b]
Cow dung	34.06 ± 1.92 [b]
Propiconazole	16.90 ± 1.53 [c]
Infected control	68.10 ± 4.68 [a]
Non-infected control	0.00 ± 0.00 [d]

(*a*) Sheath blight severity was calculated as relative lesion length (RLH) four weeks after the artificial inoculation of the pathogen, *Rhizoctonia solani*. Data present mean values ± standard error from three replicates. Values in each column followed by the same letter in superscript are not significantly ($p \leq 0.01$) different according to the Duncan's multiple range test.
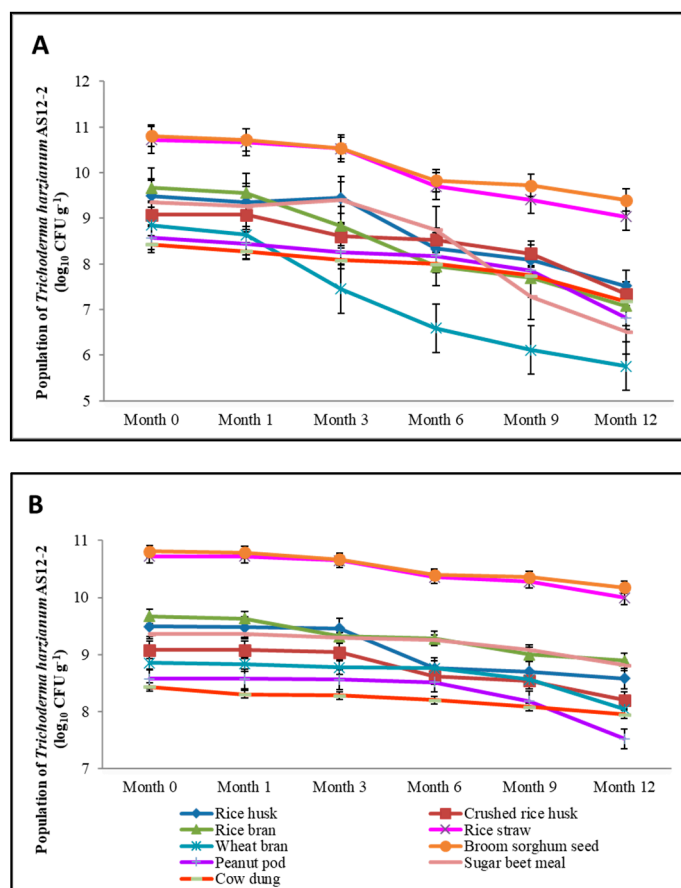
Figure 2. Viability of *Trichoderma harzianum* AS12-2 in nine organic substrates under solid-state fermentation after different incubation periods (1–12 months) at (**A**) room temperature (25 ± 2 °C) and (**B**) in the refrigerator (4 °C). Bars represent the standard error of the mean.

3.3. Bioefficacy

The results of greenhouse assay showed that all selected bioformulations, i.e., broom sorghum grain, rice husk, rice straw, sugar beet pulp and cow dung, were effective in controlling the rice sheath blight disease as compared to the control, and there was no significant difference among these five substrates ($p \leq$ 0.01). The disease severity ranged from 30.24% to 34.94% for the bioformulations, while it was 16.90% and 68.10% for the propiconazole treatment and infected control, respectively (Table 2, Figure 3).
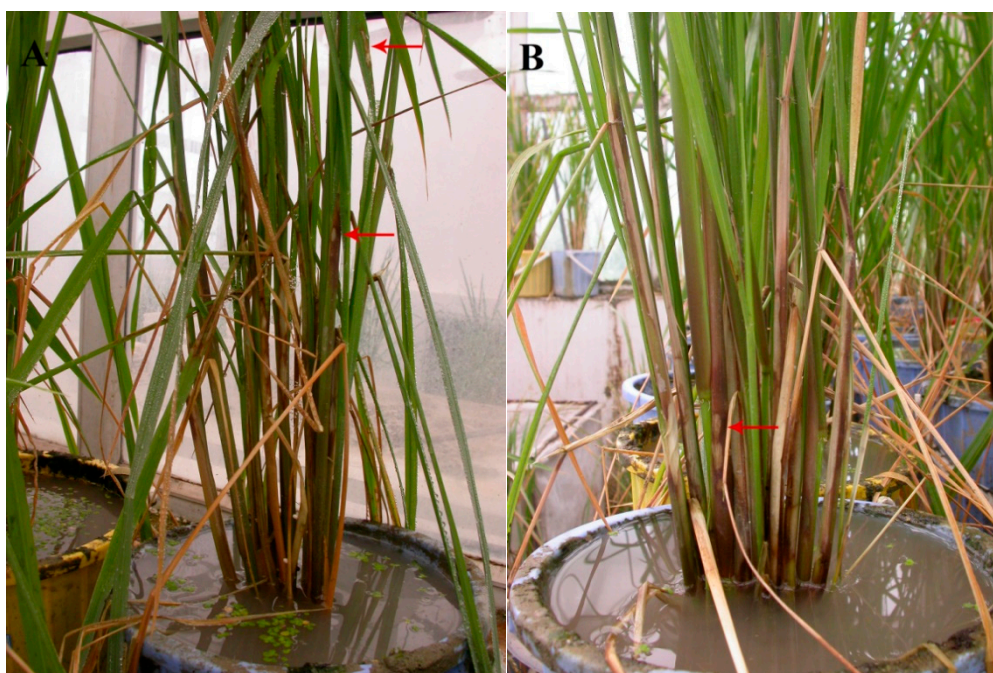
Figure 3. Lesions on rice leaves (shown by red arrows) caused by *Rhizoctonia solani*. (**A**) Infected control rice plant inoculated only with *R. solani*. Perceptible lesions are present also at higher parts of the plant, meaning a higher relative lesion length (RLH) and also implying a higher level of severity of the disease. (**B**) Rice plant inoculated with *R. solani* and *T. harzianum* AS12-2. Lesions occur at lower parts of the plant, which means a decreased RLH.

4. Discussion

Microbial biopesticides—and *Trichoderma*-based bioproducts in particular—play an important role in sustainable agricultural production. They are increasing in popularity and sales around the world owing to the changing political and social attitudes, the rise of global concern about the use of agrochemicals, the amount of investment by the large agricultural chemical companies improving products, and the growing demand for organic foods by consumers [3,8,55,75]. The establishment of innovative technology for optimizing both quantity and quality is a key factor for the manufacturing of microbial biopesticides. The success of microbial biopesticides in the crop protection market depends not only on the effective antagonists, but also on the costs involved and the method of application [5,6,8,24,39,62]. In other words, the mass production systems should be cost-effective and compatible with industrial and commercial development methods and field applications [10]. Intensive studies are needed to select proper fermentation substrates that provide massive, stable and effective microbial populations for the formulation process [76]. Nonetheless, in order to select a suitable substrate for the fermentation process, its availability and cost, as well as the amount and quality of produced conidia, should be considered. We investigated the growth and multiplication of *T. harzianum* strain AS12-2, on a whole range of different organic materials locally available in northern Iran, in SSF to select suitable agricultural wastes or agriculture by-products that can favor the production of a high amount of microbial biomass with prolonged viability. The successful commercial application of this promising biocontrol agent depends on the production of large quantities of conidia with minimum manufacturing cost. With this in mind, easily available and inexpensive biodegradable substrates were used in the present study to screen for their efficacy in producing mass inoculum of the fungal antagonist and to test its shelf life.

Members belonging to the genus *Trichoderma* are saprophytic fungi, which grow profusely on a wide range of organic substrates in nature [45]. Most commercial products contain *Trichoderma* conidia; thus, the high productivity in the SSF system is a critical factor of a successful biocontrol

agent [77]. Thangavelu et al. [78] evaluated five different organic substrates, i.e., rice bran, rice chaffy grain, farmyard manure, banana pseudostem and dried banana leaf, for SSF of a biocontrol isolate of *T. harzianum*, and reported that dried banana leaf was the best material to support the fungus growth and produced a high density of propagules. Rini and Sulochana [18] studied cow dung, neem cake, coir pith, sorghum grains, sawdust and rice bran as organic substrates for mass production of *Trichoderma* spp., and they observed the highest initial population in sorghum grains. Singh et al. [79] studied the effect of different agricultural wastes on the conidiation and shelf life of a *T. harzianum* strain, and indicated that all the substrates resulted in suitable propagation of *T. harzianum*; however, used tea leaves and wheat bran–sawdust were superior in population count and shelf life, respectively. Cavalcante et al. [42] used low-cost substrates including rice, corn, and wheat brans to produce spores of four different *Trichoderma* species, and found that wheat bran was the most suitable substrate for the conidium production of all examined *Trichoderma* strains. Sargin et al. [37] tested various inexpensive agricultural co-products, including wheat bran, sawdust, rice straw, hazelnut shell, grape marc and cotton seed cake, for propagule production of a *T. harzianum* strain, and reported that the maximum micropropagule count was achieved with a wheat bran–malt sprout mixture. Rayhane et al. [20] studied a fermentation process for enzymes and conidia with a *T. asperellum* strain and scaled-up the process from flask and glass column to a bioreactor. Fungal growth and sporulation were investigated using a mixture of vine shoots, jatropha cake, olive pomace and olive oil as substrate. The optimum production of lipases and amylases, as well as conidia (as 8.55×10^9 conidia g^{-1}), was observed in the bioreactor.

Results of the current research showed that *T. harzianum* AS12-2 grew on all 13 examined solid substrates and abundantly sporulated on them, but the level of colonization and production of biomass differed among the different growth media. This difference reflects the various ingredients in organic substrates and the food preference of the *Trichoderma* strain. The physicochemical features of organic substrates greatly affect the fermentation process [80]. The best colonization was obtained on rice husk, rice straw, broom sorghum grain, sugar beet pulp, cow dung and water fern, all of them available at very low cost. Surprisingly, rice and wheat brans, which are widely used as organic substrates for the mass production of different fungi including *Trichoderma* spp. [81–84], resulted in only moderate growth of the biocontrol strain examined in this study. One of the most important criteria for selecting a substrate for SSF is the amount of sporulation of the target microorganism. The fungal population, which is represented as CFU per gram, of a given microbial biopesticide usually affects its bioefficacy in natural systems, and is also a critical factor in quality control experiments when the biopesticides legally face registration processes. Maximum populations of *T. harzianum* AS12-2 were recorded for broom sorghum grain, rice straw, rice bran, rice husk and sugar beet pulp after one month of incubation of the substrates at room temperature. Among them, higher numbers of conidia were observed on the first two substrates, which were above 1×10^9 CFU g^{-1}. Flodman and Noureddini [23] reported 7.5×10^8 conidia g^{-1} of *T. reesei* using corn cob wastes as a substrate in an Erlenmeyer bioreactor. De la Cruz-Quiroz et al. [44] also used corn cob as a substrate and a plastic bag as a bioreactor for the solid-state fermentation of a *T. asperellum* strain, and the sporulation rate was 1.4×10^9 conidia g^{-1}. Kaushal and Chandel [85] reported that the highest population count of *Trichoderma* strains was observed in wheat straw. Rice-based substrates are the most common media for the growth and sporulation of many fungi [25]. Rice straw is rich in cellulose, hemicellulose and lignin, and represents a good source of nutrients for the prolific growth and sporulation of *Trichoderma* strains [86]. Since the biocontrol strain in this study originated from the rice field, it predictably preferred the rice-based substrate for colonization as well as conidiation. Sala et al. [25] used rice husk in the SSF of *T. harzianum* and achieved final spore concentrations up to 2.0×10^9 conidia g^{-1} dry matter. In addition, Sahayaraj and Namasivayam [87] reported that among different substrates, sorghum grain achieved the highest spore production in *Paecilomyces fumosoroseus* and *Verticillium lecanii* strains.

According to our results, the extent of colonization does not necessarily correlate with the conidiation of *T. harzianum* AS12-2 in each substrate. For example, the fungus highly colonized water

fern, but the CFU number was relatively low on this substrate. On the contrary, in the case of rice bran, despite the moderate colonization, the conidiation was very high. Mascarin et al. [88] also concluded that some substrates favor vegetative growth with low conidium production.

Apart from the CFU, the viability of the biocontrol agent and shelf life of the fungal biomass also play significant roles in successful marketing [10]. In general, the antagonists that multiply in an organic food base have a longer shelf life than in inert or inorganic food bases. In this study, the shelf life of nine air-dried substrates containing biomass of *T. harzianum* AS12-2 was examined over a period of 12 months at two temperatures. In fact, we monitored the changes of fungal population in each substrate and compared the organic substrates in terms of this trait. The population of the fungus reduced with time after storage of the substrates at room temperature, but the pattern of the decline and the steepness of the curve were different among them. In spite of the considerable viability loss, broom sorghum grain and rice straw showed a very high population at the end and were selected as the best substrates with the longest shelf life after one year of storage at room temperature. Furthermore, despite high initial population, some substrates, i.e., wheat bran and sugar beet meal, showed a considerable decrease in CFUs at Month 12. In comparison, the viability of *T. harzianum* AS12-2 in all nine substrates reduced during incubation in the refrigerator, but the population decline was modest compared to that at room temperature, and the final population was relatively high. As expected, fungal viability was highest in broom sorghum grain and rice straw compared to the other substrates. Overall, despite the similarity of patterns, the viability and population of the antagonist in all substrates tended to be less affected by storage at 4 °C than at room temperature. Reduction in the shelf life of fungal biocontrol agents during storage is consistent with the findings of other researchers, who reported the negative effects of high temperatures and long-term storage on the viability of the inocula of *Trichoderma* spp. [70,79,89] and other fungi [35,90].

The result of the bioefficacy studies showed that the biocontrol agent produced in five selected substrates significantly and effectively reduced the sheath blight severity as compared to the controls, and there was no statistically significant difference among the substrates regarding the level of disease control.

5. Conclusions

The effect of several easily available agricultural by-products was evaluated for solid-state fermentation of a biocontrol agent, and its shelf life was assessed for one year at two temperatures. The current study shows that most of the screened organic substrates can be used to ensure the quantity and quality of *T. harzianum* AS12-2 inoculum at a low cost. However, the production of a large number of highly viable spores is influenced by the type of the substrate. Although some substrates retain the viability of *T. harzianum* AS12-2 for a long period, there was a general decline in the number of CFUs in all substrates with the time of storage at both temperatures, with a rapid decrease occurring at room temperature. In other words, *T. harzianum* AS12-2 survived better at 4 °C (final populations between 3.3×10^7 and 1.0×10^{10} CFU g^{-1} substrate) than at 25 ± 2 °C (final populations between 5.8×10^5 and 2.5×10^9 CFU g^{-1} substrate) over a period of 12 months. The maximum CFUs and shelf life were obtained on broom sorghum grain, rice straw, rice husk, sugar beet pulp and cow dung, suggesting a feasible procedure of using agro-industrial wastes for the biomass production of this biocontrol agent. These substrates did not affect the biocontrol potential of *T. harzianum* AS12-2, suggesting that any of the five substrates could be selected for industrial scale SSF. Scale-up of production is an important factor in developing a laboratory-scale process. Our results suggest that the aforementioned substrates can be recommended as suitable fermentation media for the mass multiplication of *Trichoderma* strains, and that the SSF process reported here could be scaled up and developed for cost-effective commercial production of *Trichoderma*-based bio-products. Altogether, industrial-scale production could be achieved with low-cost material and equipment from low total capital investments.

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