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RESEARCH ARTICLE

Soil application of rice husk as a natural silicon source to enhance some chemical defense responses against foliar fungal pathogens and growth performance of Bitter Gourd (*Momordica charantia L.*)

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Abstract: Rice husk is a natural Silicon (Si) source. This study evaluated the effect of rice husk in two different forms; ground rice husk (GRH) and rice husk ash (RHA) on downy mildew in bitter gourd (*Momordica charantia* L.) leaves caused by *Pseudoperonospora* sp. Rice husk was added to the growing medium to achieve the final concentration of 200 mg Si /kg soil. Si accumulation in leaves, disease severity, plant growth parameters, cuticle-epidermal layer thickness, chlorophyll content, total phenolic content, peroxidase (POD) and polyphenol oxidase (PPO) activity of leaves were measured. Husk treatment lowered the downy mildew severity significantly ($p < 0.05$) in leaves although the difference between the effects of two husk forms was insignificant. Numbers of leaves, flowers and fruits in husk-treated plants were significantly higher ($p < 0.05$) than those in control plants. Cuticle-epidermal layer thickness in GRH, RHA-treated and control plants were 16.8 ± 1.21, 18.2 ± 0.98 and 13.2 ± 1.27 µm respectively. Total phenol content, POD, PPO activity and chlorophyll content in rice husk-treated plants were significantly ($p < 0.05$) higher than that of control plants. The enhanced disease resistance in rice husk-treated plants appears to be positively associated with the higher accumulation of silicon and Si-enhanced phenolic content and increased activity of PPO and POD enzymes in leaves.

Keywords: Bitter gourd, silicon, rice husk, foliar diseases.

INTRODUCTION

Rice husk (RH) is the sheath which forms the cover of rice grains during their growth. It is one of the most widely available agricultural by-products in rice producing countries throughout the world, which accounts for 20% of the annual world rice production (Battegazzore *et al*., 2014). Currently, rice husk is used for different purposes; as a fuel, as material for animal husbandry, as biofertilizer, as a raw material for construction industry etc. However, it is still an under-utilized product of rice milling and thus often burned in open air or dumped on wasteland. Rice husk ash (RHA) is produced during the combustion of rice husk. When burning temperature is not uniform, it may yield different types of RHA; black, gray or white. In fully burnt RH, silica (SiO_2) is the main component which accounts for more than 95% of total elements present (Haslinawati, *et al*., 2009). Numerous reports suggest that black to gray RHA can be effectively used in rice nurseries and in the main rice fields to achieve healthy seedlings and higher yields. According to Ishibashi (1956) carbonized RH is a good source of Silicon (Si) while unprocessed RH is not. Moreover, amendment of soil with ground RH was found to enhance the yield of cowpea (Aliyu *et al*., 2011) whereas composted RH was also useful in improving growth and some biochemical parameters including chlorophyll content, total carbohydrates and crude protein content in sunflower plant (Badar *et al*., 2014). As reported by Jayawardane *et al*. (2014), leachates from RH (which was produced by soaking RH in water) was effective in enhancing growth, yield and resistance against anthracnose disease in capsicum.

Though Si not considered as an essential element for plant growth, many beneficial effects of Si have been especially documented in 'Si-accumulator' plants like wheat, barley and rice (Ma *et al*., 2001). These include alleviation of abiotic stresses and suppression of diseases in several plant species. The Si-mediated resistance to pathogen infection has mostly been related to activation of (i) physical defense mechanisms such as formation of papillae and cuticle-Si double layer; (ii) activation of chemical defense such as defense-related enzymes and production of antimicrobial substances; and (iii) molecular mechanisms including transcryptomic and proteomic regulation in plants (Wang *et al*., 2017). Potassium silicate (K_2SiO_3), a highly soluble form of Si, has been widely studied for its beneficial effects in controlling diseases in many crops.

We previously reported that the root application of soluble Si (in the form of K_2SiO_3) reduces the severity of major foliar fungal diseases in bitter gourd (*Momordica charantia* L.) including powdery mildew caused by *Erysiphe sp.* and downy mildew caused by *Pseudoperonospora sp.* (Ratnayake *et al*., 2014, 2015, 2016a and 2016b). The reduction of disease severity could partly be attributed to Si-enhanced chemical defense responses including elevated activity of enzymes; peroxidase, polyphenol oxidase and pathogenesis-related proteins; chitinase and β-1, 3-glucanase in leaf tissues (Ratnayake *et al*., 2016c). In this context, the aims of the current work were to ascertain

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the effectiveness of soil amendment with rice husk as a natural Si source in controlling foliar fungal diseases, and the effects on some growth and yield parameters of bitter gourd plant.

MATERIALS AND METHODS

Plant material and growth media

Momordica charantia L. cultivar 'Matale green' plants were grown on plastic pots containing sand: top soil: compost at 2:1:1 ratio as the growth medium. The concentration of available Si in this medium was 2.9 mg/L, as assayed by colorimetry (Snyder, 2001) after extraction with Na_2CO_3/NH_4NO_3 (Buck *et al*., 2010). The pots were arranged in a plant house at the Department of Botany, University of Peradeniya, Sri Lanka (7.0 - 9.0º N; 81 - 82º E) with 1.5 m x 1.5 m plant spacing. Rice husk was used in two different forms viz. ground rice husk (GRH), which was obtained by grinding the rice husk on laboratory scale grinder and rice husk ash (RHA),which was obtained by burning in a Muffle furnace at 500°C for 5 hours. The plant available Si in each form of husk was quantified before adding to the growth media. The husk was added to the growth media to achieve a final concentration of 200 ppm Si/kg (plant available Si). This 200 ppm Si/kg soil has been identified previously by us as the best Si concentration to be used on bitter gourd to control foliar fungal diseases (Ratnayake *et al*., 2016a). Amendment of growth media with RH was started at the four leaf stage of plantlets and continued up to 28 days at seven- day intervals. Plants grown on media without rice husk served as controls. Each treatment consisted of 20 replicate plants and the experiment was repeated twice.

Plant-available Si content in rice husk

Soluble Si contents in GRH and RHA were measured by colorimetric analysis (Snyder, 2001) after extraction using the method of Nonaka and Takahashi (1988). Ten grams (10 g) of each sample was mixed in 60 ml of water and shaken for 1 hour, then kept undisturbed for one week at 40 ºC before determination of soluble Si content.

Total Si levels in leaves along plant growth

A time course analysis of the total soluble Si levels in leaves from different treatments was done starting at 35 days after the first dose of husk application and continued up to 77 days at seven-day intervals (Rice husk was applied up to 28 days, and Si analysis was started at 35 days. Our observations revealed that initial fruit set occurs at 70-77 days after the first Si treatment. Therefore data were taken up to 77 days).

Extraction and analysis of Si were as described by us recently (Ratnayake *et al*., 2016a). Five leaves, between 15^{th} - 60^{th} nodes from each plant, were oven dried at 70 ºC for three days and the Si contents of the powdered and autoclave-digested samples were determined by colorimetric analysis (Snyder, 2001).

Plant growth

Number of leaves, flowers, and fruits per plant were counted at seven days interval starting from 35 days after the first husk application.

Disease severity in leaves

Severity of downy mildew disease (the most common disease during the period of investigation) in leaves was rated separately through external observations on weekly basis using a self-prepared scale (0 = no disease, 1 = 1 to 20%, 2 = 21 to 40%, 3 = 41 to 60%, 4 = 61 to 80%, and 5 = 81 to 100% of upper leaf surface covered with disease) (Ratnayake *et al*., 2016b). As all the treated and control plants were arranged randomly in the field and fertilization, watering and other field practices were applied similarly to each pot, it was assumed that all the plants got identical level of inoculums.

Structural changes in leaves

Transverse sections (TS) of leaves obtained from the plants with different treatments were observed under light microscope and cuticle–epidermal layer thickness was measured. Prior to the appearance of disease, 42 days after the first dose of husk application, 5 leaves between 5^{th}-10^{th} node of each plant were tagged, and randomly selected 10 leaves from the tagged leaves were removed per treatment for anatomical study.

Total phenolic content, peroxidase (POD) and polyphenol oxidase (PPO) activity of leaves

Preparation of leaf samples

Thirty five days after the first husk application, three to four leaves between 10^{th}-15^{th} node of the plant were selected and their fresh weights were noted before extraction for enzyme analysis.

Total phenolic content of leaves

Leaf samples were extracted for phenolic compounds with 80% (v/v) methanol (Torti *et al*., 1995) and the total phenolic content was determined using Folin–Ciocalteu assay using gallic acid as the phenolic standard (Singleton *et al*., 1999). Absorbance was measured on a Spectrophotometer (Thermo Spectronic) at 760 nm against methanol blank. Total phenolic content was expressed in mg of gallic acid equivalents (GAE) /100 g (dry weight) of leaf.

Peroxidase (POD) assay

The POD activity of the leaf extract was determined as described recently for bitter gourd (Ratnayke *et al*., 2016c) by measuring the appearance of pink/brown colour resulting from guaiacol oxidation in the presence of hydrogen peroxide (Dann and Deverall, 2000). Appropriately diluted plant extract (50 or 100 µL) was added to the reaction mixture and the absorbance was measured on a Spectrophotometer at 470 nm every 10 s for 60 s. The activity of POD was detected as change in absorbance at 470 nm and expressed in min^{-1} mg $protein^{-1}$.

Polyphenol oxidase (PPO) assay

Method used to extract PPO was based on our previous experiments (Ratnayake *et al*., 2016c). This method involved spectrophotometric measurement of the change in intensity of a dark coloured polymeric compound resulted from oxidation of catechol of the reaction mixture upon adding leaf extracts. The enzyme activity in the plant extract was expressed in min^{-1} mg protein $^{-1}$ upon calculating the change in absorbance at 400 nm (Cheriff *et al*., 1994).

Chlorophyll content of leaves

Two leaves excised at 10^{th} -12^{th} nodes of the vine per replicate plant at 35 days after first husk application were used. Prior to extraction, leaf samples were cleaned with deionized water to remove any surface contamination. Five grams (5 g) of fresh leaf tissue was extracted with 80% acetone, filtered and the absorbance was measured at 643 nm and 660 nm on a Spectrophotometer. Chlorophyll content was calculated according to the method of AOAC (2000).

Data analysis

Data were analyzed using ANOVA using SAS statistical software for windows version 9.0. Differences between treatment means were obtained by DMRT at $p < 0.05$.

RESULTS AND DISCUSSION

Plant-available Si content in rice husk

As measured by spectrophotometry, plant-available (soluble) Si content in RHA (56.1 ± 2.1%) was significantly greater ($p<0.05$) than that in GRH (18.2 ± 2.4%). Thus the amount of RHA needed to achieve 200 ppm (0.36g/kg soil) was lesser than that of GRH (1.1g/kg soil). High processing temperatures regularly release Si from tightly bound state and as a result, solubility increases (Gascho, 2001). This may be due to the increased level of soluble Si in RHA. Beneficial effects of carbonized rice husk (CRH; partially burnt RH) on plant growth had also been reported (Haefele *et al*., 2011). However, our preliminary investigations revealed that the presence of carbonized rice husk (partially burnt) in growth media (1g/kg soil) hinder the growth of bitter gourd plants (data not shown). It may have resulted from the changes resulted by burned husk on pH and especially due to increased C/N ratio in soil. The property/form of rice husk added to soil appears to determine the overall performance of plant through their effects on soil hydro-physical properties. The changes in soil in turn neutralize the soil acidity and increase nutrient availability to plants (Sandrini, 2010). The magnitude of these effects depends on the characteristics of rice husk.

Si accumulation in leaves along plant growth

All rice husk treated plants exhibited significantly higher ($p<0.05$) Si levels in leaves compared to those from non-treated plants. Both GRH and RHA were equally effective in elevating the total Si contents of leaves. A gradual increase in total Si content up to 42 days was observed in husk-treated plants. About 70-77 days after first husk application, Si contents in leaves dropped to the levels similar to those in non-treated controls (Figure 1).

Disease severity in leaves

Downy mildew, most common disease during the study period was identified by its typical symptoms (Kuepper, 2003). The causative fungus was identified as a *Pseudoperonospora* sp. based on fungal morphological characteristics including lemon shaped conidia borne at the branched pointed tips of sporangiophores (Palti, 1975). Symptoms started to appear first in control plants by 35 days after seedling emergence whereas disease appearance was delayed by 9 days in rice husk-treated plants (Figure 2). It was interesting to note that, disease severity of rice husk treated plants was significantly lower ($P<0.05$) than that of control plants during the entire period of experiment

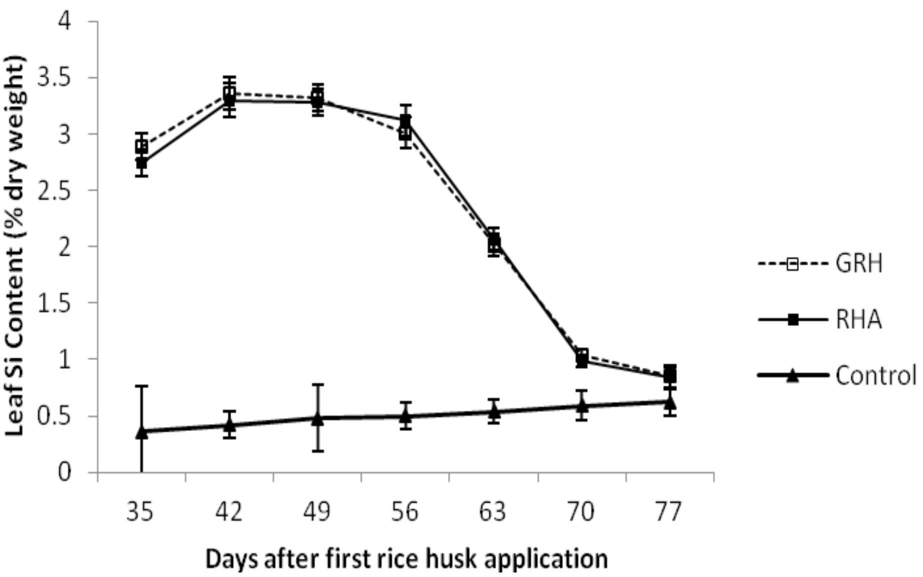

Figure 1: Changes in Si accumulation in bitter gourd leaves over time. (Husk application was started at 4 leaf stage and continued up to 28 days at seven-day intervals). (GRH = ground rice husk; RHA = rice husk ash).

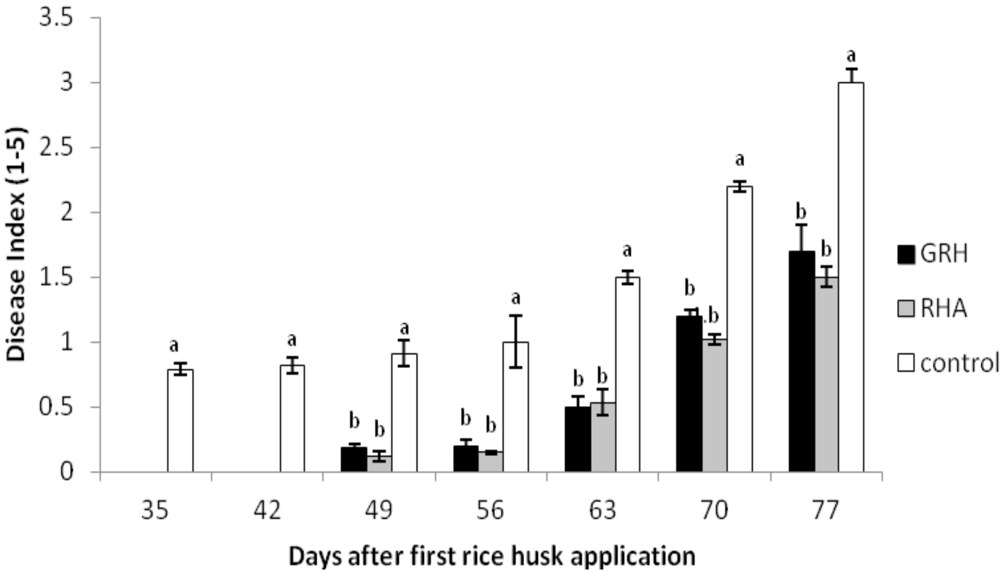

Figure 2: Variation of the disease severity on bitter gourd leaves due to the different silicon concentrations. Different letters above bars under each day indicates significant difference at P < 0.05. (GRH = Ground rice husk; RHA = Rice husk ash). Disease index 0 = No disease, 5 = 81-100% of upper leaf surface covered with the disease).

Table 1: Variation in leaf, flower and fruit number per bitter gourd plant with different rice husk treatments.

Treatment	Leaves	Flowers	Fruits
GRH	83.2[a]	6.86[b]	3.68[a]
RHA	82.4[a]	7.18[a]	3.74[a]
Control	78.4[b]	5.95[c]	3.12[b]

Observations made at 70 days after first husk application (GRH = ground rice husk; RHA = rice husk ash). Mean values followed by different letters under each column are significantly different at P < 0.05.

and both GRH and RHA were equally effective in reducing the disease severity. A clear reduction in downy mildew severity and levels of leaf accumulated Si was reported recently for bitter gourd plants grown in potassium silicate-amended media (Ratnayake *et al.*, 2016b). The reduction of disease severity and levels of foliar accumulated Si in plants grown on rice husk-amended media were comparable with those grown in potassium silicate- amended media.

Plant growth and chlorophyll content of leaves

A significantly higher (P<0.05) numbers of leaves, flowers and fruits per plant were observed in husk-treated plants (irrespective of the form of husk used) compared to control plants (Table 1). Total chlorophyll contents in husk-treated plants were also significantly higher (P<0.05) than that of control plants (Table 2). Improved growth characteristics in plants grown in husk-amended media also could be attributed to increased availability of soluble Si to plants. This observation agrees with the findings that root application of potassium silicate improves growth and yield parameters of bitter gourd plant (Ratnayake *et al.*, 2016c). A positive correlation between soluble Si application and enhanced productivity has been reported for many crops such as barley, corn, chili, cucumber, tomato, sugarcane and wheat (Meena *et al.*, 2014; Jayawardana *et al.*, 2016). Elevated chlorophyll levels may have partly contributed to the increased yield of bitter gourd plants. Si applications have reported to enhance photosynthetic efficiency by enhancing tiller and leaf formation in several crops (Meena *et al.*, 2014). Moreover, Si-induced strengthening of the leaf prevents leaf drooping thus contributing to enhanced light capturing capacity during photosynthesis (Donega *et al.*, 2009). Zhu *et al.* (2004) observed that Si - induced increase in total chlorophyll content in leaves can also be related to the extent of thylakoid stacking.

An increased uptake of N levels was observed in bitter gourd plants grown in husk-amended media (Ratnayake *et al.*, 2017). Si has the ability to increase the levels of some nutrients such as N and P thus resulting boosted yields of many cereal crops including rice (Singh *et al.*, 2006). However, a reduction in N uptake has also been reported for certain crops with Si application (Horiguchi, 1988).

Structural changes in leaves

The thickness of the cuticle - epidermal layer in transverse sections of leaves in rice husk-treated plants was significantly higher (P<0.05) than that of the control plants. The highest thickness was observed in RHA-treated plants (18.2±0.98µm) compared to that in GRH-treated plants (16.8 ± 1.21µm) and non-treated plants (13.2 ± 1.27µm). The increased cuticle-epidermal layer thickness of bitter

Table 2: Effect of ground rice husk (GRH) and Rice husk ash (RHA) on chlorophyll contents in bitter gourd leaves.

Type of Chlorophyll (mg/L)*	RHA	GRH	Control
Chlorophyll a	14.47 ± 0.28[a]	14.53 ± 0.94[a]	9.12 ± 0.76[b]
Chlorophyll b	8.61 ±1.34[a]	8.64 ± 0.98[a]	5.98 ± 1.32[a]
Total Chlorophyll	22.94± 1.64[a]	22.87± 1.98[a]	15.94± 2.31[b]

*Data represent the analysis of leaf tissues on 70th day after first husk application. Mean values followed by different letters under each row are significantly different at $P < 0.05$. RHA = rice husk ash GRH = ground raw rice husk.

Table 3: Effect of rice husk ash (RHA) and ground raw rice husk (GRH) on total phenol content, activities of peroxidase and polyphenol oxidase enzymes in bitter gourd leaves.

Parameter	GRH	RHA	Control
Total Phenol (mg GAE/100 g leaf dw)	150.32[b]	153.20[a]	135.51[c]
POD activity (Δ absorbance at 400 nm min^{-1} mg $protein^{-1}$)	2.060[b]	2.340[a]	1.840[c]
PPO activity (Δ absorbance at 400 nm min^{-1} mg $protein^{-1}$)	0.029[a]	0.031[a]	0.022[b]

Mean values followed by different letters under each row are significantly different at $P < 0.05$.
(GRH =Ground rice husk; RHA =Rice husk ash).

gourd plants raised on husk-amended media may have contributed partly to their enhanced resistance to fungal disease. Si in the solution of growth medium is absorbed in to the plant in the form of monosilicic acid (H_4SiO_4) and is transported from root to shoot through the water current in the xylem (Ma *et al*., 2003). Once transported to the leaves through transpiration stream, monosilicic acid can get concentrated beneath the cuticle in the leaf epidermal cells thus forming double-cuticular layer. Further increase of soluble Si levels in leaves may lead monosilicic acid to polymerize into amorphous SiO_2 (Yoshida, 1975). Si deposited on the epidermis provides an additional barrier to pathogen invasion thereby making the plants more resistant to diseases (Heine *et al*., 2007).

Total phenolic content of leaves

Total phenol contents, PPO and POD enzyme activities in leaves from rice husk- treated plants were significantly higher than those of control plants. RHA was more effective than GRH in elevating the total phenol content and POD activity (Table 3). The progress of infection leads to a range of defense responses in the host. Various antimicrobial compounds in plants such as phenols are involved in early defense responses (Osbourn, 1996). Peroxidases and other phenol oxidizing enzymes convert phenolics to more toxic forms that are inhibitory to the pathogenic growth (Kuvalekar *et al*., 2011). Thus, POD and PPO play an important role in plant stress caused by different stimuli including fungal pathogens, wounding or abiotic stress. Increased epidermal-cuticle layer thickness observed in this study may partly be related to the increased level of POD activity that has a specific role in lignification and strengthening the plant cell wall that is highly resistant to biodegradation (Schoemaker and Piontek 1996; Lee *et al*., 2007; Jaiti *et al*., 2009). Further, PPO contributes to lignification (Ralph *et al*., 2008) and together with POD it consumes oxygen and produces quinones, which may be toxic to pathogens. Therefore, decrease in disease progress in rice husk treated plants suggests the relationship with phenols and tested enzymes in the study. On the other hand, impregnation of epidermal cell walls with silica in rise husk-amended plants may also have made the initial penetration difficult for the downy mildew pathogen (Heath and Stumf, 1986). Furthermore, silica treatment provides resistance to mildew fungi through papillae formation and deposition of callose in the epidermal cell walls (Bélanger *et al*., 2003). Defense against direct-penetrating fungi including biotrophs causing rust and mildews is more successful when they fail to penetrate the epidermal cell wall, the first line of defense in the host plant (Heath, 2002).

When comparing with our previous observations with potassium silicate effects on bitter gourd (Ratnayake *et al.*, 2016c), it was clear that foliar disease severity gradually decreased after the husk application was stopped. Once Si is deposited in a plant tissue in the polymerized form it is not remobilized within the plant (Raven, 1983). Presence of soluble Si in plant tissues is necessary to maintain the disease resistance against pathogens. Therefore, depletion of soluble Si from growth media may lead to gradual loss of plant resistance to pathogens. In addition to the resistance mechanisms described above, Si-induced fungitoxic activity also may play a role in disease resistance as observed previously with bitter gourd grown on potassium silicate-amended media (Ratnayake *et al.,* 2016c). A positive relationship between Si-induced fungitoxic compounds and resistance against fungal diseases has been reported for many other horticultural crops (Van Bockhaven *et al*., 2013).

When considering the interaction between the biotrophic pathogen and a host plant, haustoria play a vital role as highly specialized structures through which nutrients are uptaken from the host cells. The pathogen haustorium does not have a direct contact with the host cytoplasm and the nutrient uptake takes place via an 'extracellular matrix' which encircles the haustorium. This matrix also acts as a buffer protecting the pathogen from toxins and enzymatic hydrolysis in the course of plant defense responses (Voegele and Mendgen, 2003). Therefore, further investigation is necessary for revealing the mechanism/s underlying the effects of Si-enhanced fungitoxic compounds on downy mildew pathogen on bitter gourd.

CONCLUSION

Rice husk in raw or ash form improves growth and yield characters and enhances resistance to foliar fungal pathogens in bitter gourd. The enhanced disease resistance in rice husk-treated plants appears to be positively associated with the higher accumulation of silicon and Si-induced phenolic content and increased activity of PPO and POD enzymes in leaves. Rice husk ash (RHA) can be recommended as more effective and easy-to-use form of husk than ground raw husk (GRH) that can be incorporated into potting mixtures and agricultural fields. Rice husk can be suggested as an eco-friendly low cost alternative to fungicides with several added beneficial effects that can be used in bitter gourd cultivation.

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