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Research article

Enhancing Napier grass growth under greenhouse conditions using *Bacillus* and *Trichoderma* bioproducts

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Abstract

Importance of the Work: Sustainable forage production was enhanced by addressing the limited data on microbial bioproduct effects in Napier grass cultivation.

Objectives: To evaluate the effects of *Bacillus* and *Trichoderma* bioproducts on Napier grass growth, nutrient uptake and physiological responses.

Materials and Methods: Two Napier grass cultivars were treated under greenhouse conditions with powdered *Bacillus* or liquid *T. asperellum* formulations. Growth parameters, nutrient content, chlorophyll, enzyme activities and indole-3-acetic acid (IAA) content were measured. Treatments were applied at varying frequencies and concentrations, with statistical analyses performed based on analysis of variance and t tests.

Results: The microbial bioproducts significantly ($p < 0.05$) enhanced the growth and physiological performance of Napier grass under greenhouse conditions. *Bacillus* and *Trichoderma* increased plant height and leaf number, with responses varying by cultivar and plant age. *Bacillus* improved the chlorophyll content, particularly in Pak Chong 1, while *Trichoderma* consistently promoted leaf development. Fresh biomass gains were significant only in the Taiwan treated with *Bacillus*. The enzyme activity analysis confirmed that *Trichoderma* stimulated β-1,3-glucanase and peroxidase, whereas *Bacillus* elevated IAA, indicating distinct microbial effects on plant physiology.

Main Finding: Microbial bioproducts, particularly *Bacillus* and *T. asperellum*, improved the growth and physiology of Napier grass in a cultivar-specific manner, with *Bacillus* enhancing the chlorophyll and IAA contents and *Trichoderma* stimulating β-1,3-glucanase and peroxidase activities.

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Introduction

Napier grass (*Pennisetum purpureum* Schumach) is recognized widely for its extensive use as animal feed and its superior yield per area compared to other forage grasses (Islam et al., 2024a). Additionally, it serves as a bioenergy crop capable of efficiently converting biomass into electricity (Kubde et al., 2023) Furthermore, its cultivation contributes to environmental sustainability through soil improvement and water purification functions, particularly in tropical and subtropical regions where it is well-adapted (Islam et al., 2024a). Napier grass cultivation has expanded globally in response to increasing demand for carbon-neutral biomass alternatives to fossil fuels, particularly in warm climates where, even under suboptimal management, the crop maintains high productivity (Waramit and Chaugool, 2014).

Hybrid Napier grass (*Pennisetum purpureum* × *Pennisetum americanum*), particularly the cultivar Pakchong 1, is widely cultivated in Thailand due to its high biomass yield and protein content (Onjai-uea et al., 2022; Phetsuwan et al., 2023). This C4 grass is a result of crossing pearl millet with Napier grass, offering advantages such as increased yield, better resistance to pests and diseases and enhanced tolerance to drought and flooding (Hari and Jindal, 2008; Jade et al., 2021; Mohamand et al., 2022) It is one of the most productive forage grasses globally, with Super Napier varieties capable of producing up to 20 tonnes of dry matter per hectare, harvestable 7–8 times annually (Haegele and Arjharn, 2017; Elehinafe et al., 2021).

Despite these benefits, Napier grass cultivation faces important challenges from plant diseases. Head smut, caused by *Ustilago kamerunensis* and Napier grass stunt disease, caused by the phytoplasma *Candidatus Phytoplasma oryzae*, can substantially reduce productivity (Abeysinghe et al., 2016; Wambua et al., 2017). Effective management of these diseases is difficult because their transmission involves insect vectors, complicating control efforts (Mwendia et al., 2007; Kawube et al., 2015; Asudi et al., 2017).

Recent studies have explored the potential of beneficial rhizosphere microorganisms to promote plant growth and suppress plant diseases (Roy et al., 2024; Yang et al., 2024a). They reported that the application of microbial fertilizers contributed to long-term soil health by boosting microbial diversity, supporting ecological balance and improving soil structure. For example, plant root exudates attract beneficial fungi that produce polysaccharides, which bind soil particles, enhance soil stability and create favorable conditions for plant development (Wei et al., 2024). Additionally, certain microbes enhance plant tolerance to abiotic stress, such as drought, salinity and heavy metals, through the production of stress-related compounds and gene regulation (Adedayo and Babalola, 2023; Akbari et al., 2024). Beyond growth promotion, these bioproducts play a crucial role in disease suppression. For example, beneficial microbes can produce antibiotics, compete with pathogens for space and nutrients and trigger systemic resistance in host plants, thereby enhancing plant defense mechanisms (Larran et al., 2020; Asghar et al., 2024; Islam et al., 2024b).

Antagonistic bioproducts, particularly those containing *Bacillus* spp. and *Trichoderma* spp., can enhance plant growth and suppress diseases through multiple mechanisms. For example, *Bacillus* species function as plant growth-promoting rhizobacteria (PGPR), improving nutrient availability, fixing atmospheric nitrogen (Awasthi, et al., 2011), as well as solubilizing phosphate and producing phytohormones, such as indole-3-acetic acid (IAA), to stimulate root development (Bader et al., 2020; Elsoud et al., 2023; Figueredo et al., 2023). In addition, they protect plants by producing antibiotics, competing for resources and inducing systemic resistance (Idris et al., 2007; Wang et al., 2022; Ding et al., 2024). Two *Bacillus* strains used, *B. amyloliquefaciens* and *B. velezensis*, are well-known PGPR with biocontrol potential. Notably, they promote plant growth by producing IAA, solubilizing phosphate and secreting hydrolytic enzymes that enhance nutrient availability and root development; in addition they both produce antimicrobial lipopeptides (iturin, fengycin, surfactin) and form biofilms, contributing to suppression of phytopathogens and persistence in the rhizosphere (Fan et al., 2017; Silva et al., 2019; Berber et al., 2020; Bedlovičová et al., 2021; Soliman et al., 2022).

Trichoderma asperellum is a well-known biocontrol fungus with multiple mechanisms that contribute to plant health against phytopathogens via mycoparasitism, secretion of cell wall-degrading enzymes (such as chitinases and glucanases) and the induction of plant defense responses (Carsolio et al., 1999; Kubicek et al., 2011; Tyśkiewicz, et al., 2022). Furthermore, they promote seedling vigor, root growth and soil structure, contributing to long-term soil health and stress tolerance (Nawrocka and Małolepsza, 2013; Adedayo and Babalola, 2023). In addition, species of both *Bacillus* and *Trichoderma* are known to solubilize phosphate, further supporting plant growth (Liu et al., 2021; Bedine et al., 2022). *Trichoderma* and *Bacillus* species are well-recognized biocontrol agents, partly due to their ability to produce β-1,3-glucanase key hydrolytic enzymes that degrade β-1,3-glucans in fungal cell walls, leading to pathogen lysis and inhibition. For example, in *Trichoderma* spp., such as *T. harzianum*, β-1,3-glucanase plays a critical role in mycoparasitism and in suppressing pathogens often acting synergistically with chitinase to enhance antifungal efficacy (Ramada et al., 2010; Kaur et al., 2020; Sahouli et al., 2020). Similarly, *Bacillus* spp., including *B. subtilis* and *B. amyloliquefaciens*, secrete β-1,3-glucanases with demonstrated antifungal activity against diverse plant pathogens (Thakaew and Niamsup, 2013; Zalila-Kolsi et al., 2017; Saini et al., 2024). Beyond direct antagonism, both genera can induce systemic acquired resistance, upregulating defense-related enzymes such as phenylalanine ammonia-lyase (PAL) and peroxidase (POX), as well as pathogenesis-related proteins, including β-1,3-glucanases, thereby integrating direct pathogen suppression with enhanced host defense (Konappa et al., 2016; Rajninec et al., 2021).

Therefore, the current study aimed to evaluate the effects of *Bacillus* spp. and *T. asperellum*, applied individually, on the growth of two Napier grass cultivars under greenhouse conditions, to identify the potential of these two bacteria for sustainable and productive forage cultivation.

Materials and Methods

Preparation of microorganisms

Two formulations of biocontrol agents were used: 1) a powdered formulation of mixed *Bacillus* strains, where this water-soluble product contained *Bacillus amyloliquefaciens* KPS46 and *B. velezensis* KN and was packaged in sealed aluminum foil sachets by the Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand; and 2) a liquid formulation of *T. asperellum* that was prepared at a concentration of 1 × 10^8 spores/mL by the Department of Plant Pathology, Faculty of Agriculture, Kamphaeng Saen Campus, Kasetsart University, Bangkok, Thailand.

Re-culturing of microorganisms

Both products were re-cultured on potato dextrose agar (PDA) to verify the viability and consistency of the biocontrol agents. The *Bacillus* mixture was suspended at 7 g/L and streaked onto PDA using a sterile loop. *T. asperellum* was applied at 5 µL per plate. The plates were incubated at room temperature for 7 d to observe colony growth and morphology.

Co-culture assay for antagonistic interaction

PDA plates were prepared and solidified in Petri dishes. Using a sterile cork borer, two wells (each 5 mm diameter) were created 2 cm from opposite edges. The treatments consisted of *Bacillus* suspensions (*Bacillus amyloliquefaciens* KPS46 and *B. velezensis* KN) at 0 (control), 3.5 g/L (~1.75 × 10^6 CFU/mL) or 7.0 g/L (~3.5 × 10^6 CFU/mL), co-cultured with *T. asperellum* suspensions at 0 (control), 1 × 10^4 or 1 × 10^8 spores/mL (50 µL per well). Bacterial inocula were prepared from both fresh culture suspensions and formulated powder products. For the fresh cultures of *B. amyloliquefaciens* KPS46 and *B. velezensis* KN, the working concentration was adjusted to approximately 1 × 10^8 CFU/mL based on optical density calibration and viable counts (Buensanteai et al., 2009). For the formulated powder preparations, a recommended working concentration of approximately 1 × 10^6 CFU/mL was used, following practices commonly applied in bioinoculant formulations. These concentrations were selected to represent low and high inoculum levels for direct comparison between bacterial and fungal populations, as *Trichoderma* spp. spore densities around 1 × 10^6 spores/mL have been reported as effective for plant growth promotion (Gateta et al., 2023). The plates were incubated at room temperature for 5 d and the colony diameters were measured using digital calipers. Each treatment was replicated seven times.

Chitinase enzyme activity assay was conducted based on PDA containing 2.4% colloidal chitin. *Bacillus* suspension (3.5 g/L or 7 g/L) and *T. asperellum* (1 × 10^4 or 1 × 10^8 spores/mL) were inoculated (50 µL per well at 5 mm diameter). Plates were incubated for 5 d, then flooded with 0.1% Congo red for 30 min. Plates were washed twice with 1 M NaCl. Clear zones around colonies indicated chitin degradation. Each treatment was replicated seven times (modified from Roberts and Selitrenkoff, 1985).

The cellulase enzyme activity assay involved preparing carboxymethyl cellulose (CMC) agar using: 2 g $NaNO_3$, 1 g KH_2PO_4, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.2 g peptone, 2 g CMC, 17 g agar and 1 L distilled water. After autoclaving and solidification, 5 mm wells were made. *Bacillus* suspension (3.5 or 7 g/L) and *T. asperellum* (1 × 10^4 or 1 × 10^8 spores/mL) were added at 50 µL per well. Plates were incubated at room temperature for 5 d. Post-incubation, iodine-potassium iodide solution was applied to assess cellulose degradation. Clear zones indicated cellulase activity (Hankin et al., 1971). Each treatment was repeated seven times.

These enzymes were assayed as indicators of the biocontrol potential of the bioagents, which may indirectly contribute to plant growth promotion through pathogen suppression and improved root colonization.

Greenhouse Trial I: Growth performance and leaf nutrient content of Napier grass treated with mixed Bacillus strains and T. asperellum

Organic Napier grass seedlings of the two cultivars (Pak Chong 1 and Taiwan) were transplanted into 30–cm–diameter pots filled with a compost-to-soil ratio of 3:1. The plants were maintained in a greenhouse under average day/night temperatures of 28 ± 2°C, relative humidity of 65–75% and natural daylight averaging 12 hr. Light intensity was not artificially controlled; however, the greenhouse structure ensured uniform distribution of natural light. Plants were watered every 2 d throughout the experimental period. After 4 mth of growth, three different soil drenching treatments were applied: Treatment 1 (T1) consisted of mixed *Bacillus* strains (20 g/3L), applied at 250 mL per pot every 7 d for a total of four applications (7 d interval × 4); Treatment 2 (T2) used the same *Bacillus* concentration, applied at 250 mL per pot every 14 d for two applications (14 d interval × 2); and Treatment 3 (T3) involved the application of *T. asperellum* (1×10^8 spores/mL) at 5 mL/L, applied at 250 mL per pot every 14 d for two applications (14 d interval × 2). The control group (T4) received no microbial treatment. Each treatment was replicated eight times. At age 5 mth, plant growth was recorded based on: plant height (measured from the soil surface to the tip of the tallest leaf), stem diameter (measured at the base of the stem) and the number of fully expanded leaves per plant. Leaf samples were analyzed for nutrient contents—nitrogen (N), phosphorus (P) and potassium (K).

For the nutrient content analysis, leaf samples were collected and analyzed at the Department of Soil Science, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand. The total N content was determined using the Kjeldahl method following digestion with a sulfuric acid-sodium sulfate-selenium mixture, as described by Jackson (1965). Total P was analyzed after tri-acid digestion with nitric acid (HNO_3), sulfuric acid (H_2SO_4) and perchloric acid ($HClO_4$), using the Vanado-molybdate yellow colorimetric method, according to Johnson and Ulrich (1959). The total K content was also determined following tri-acid digestion, with the concentration being measured using atomic absorption spectrophotometry, following the method of Watson and Isaac (1990).

Greenhouse Trial II: Evaluation of growth, yield, chlorophyll content, enzyme activities and indole-3-acetic acid accumulation in Napier grass treated with mixed Bacillus strains and T. asperellum

Seedlings aged 1 mth of the two Napier grass cultivars, Pak Chong 1 and Taiwan, were transplanted into pots (30 cm diameter) containing a compost-to-soil mixture (3:1, volume per volume). The greenhouse conditions were the same as described in Greenhouse Trial I (28 ± 2°C, relative humidity of 65–75% and natural daylight averaging 12 h). Plants were subjected to three treatments: (T1) application of mixed *Bacillus* strains (20 g/3 L of water) at 250 mL per pot every 14 d for a total of two applications (14 d interval × 2); (T2) application of *T. asperellum* (1×10^8 spores/mL) at 5 mL/L, applied at 250 mL per pot every 14 d for two applications (14 d interval × 2); and (T3) an untreated control group.

Growth and yield data were collected 1 mth after the final treatment. Plant height (in centimeters) was measured from the soil surface to the tip of the tallest leaf to assess vertical growth. Stem diameter (in centimeters) was measured at the base of the main stem to evaluate structural development. The number of fully expanded leaves per plant was counted to determine differences in foliage production. Fresh weight (in grams) was recorded to evaluate biomass accumulation, while dry weight (in grams) was determined after drying the samples in an oven at 70°C for 48 hr. These parameters were compared with the control group to assess the effects of the microbial treatments on biomass production and water retention. Measurements were conducted at 1 mth, 2 mth and 3 mth after treatment, with eight replicates per treatment.

The chlorophyll content was measured in SPAD units using a SPAD-502 Plus chlorophyll meter (Konica Minolta). Readings were taken from the second fully expanded leaf from the top of each plant. Measurements were conducted at 2 mth and 3 mth after treatment, with eight replicates per treatment.

For enzyme activity assays, leaf samples were collected and immediately stored on ice. Total protein was extracted by homogenizing 0.15 g of fresh leaf tissue in 1.5 mL of extraction buffer containing Tris-HCl, KCl, a proteinase inhibitor, Triton X-100 and polyvinylpolypyrrolidone. Protein concentrations were quantified using the method of Bradford (1976), based on measuring absorbance at 595 nm.

Analysis of PAL activity was determined by mixing 400 µL of protein extract with 704 µL of 50 mM Tris buffer (pH 8.8) and 280 µL of 12 mM L-phenylalanine. The reaction mixture was incubated at 40°C for 30 min and terminated using 800 µL of 2 M HCl. Absorbance was measured at 290 nm and the PAL activity was calculated using Equation 1:

$$\text{PAL activity} = (0.0028 \times \text{Absorbance}_{290}) + 0.2041 \qquad (1)$$

The protein content was calculated according to (Tomoiagă et al., 2020) using Equation 2:

$$\text{Protein content} = (0.0015 \times \text{Absorbance}_{595}) - 0.0389 \qquad (2)$$

Analysis of the POX enzyme activity was determined from Napier grass leaf samples extracted in homogenization buffer, using 100 µL of extract per assay. The reaction mixture consisted of 125 µL of guaiacol, 153 µL of hydrogen peroxide and 10 µL of 50 mM sodium phosphate buffer (pH 6.0). The reaction was initiated by adding the extract to the substrate solution, followed by measuring the absorbance at 470 nm using a spectrophotometer (Shimadzu UV-1800, Shimadzu Corporation, Japan). Readings were recorded every 30 s for a total duration of 2 min. Enzyme activity was calculated using the average change in absorbance over time according to the method of Chagas et al. (2008) using Equation 3:

$$\text{POX activity} = [(A_{60} - A_0) + (A_{90} - A_{30}) + (A_{120} - A_{60}] / 3 \qquad (3)$$

where A_{xx} is the absorbance measured at 470 nm at time xx s.

The total protein content was determined using the method of Bradford (1976) and calculated using Equation 4:

$$\text{Total protein} = (0.0015 \times \text{Absorbance}_{595}) - 0.0389 \qquad (4)$$

Analysis of the β-1,3-glucanase enzyme activity was determined using Napier grass leaf samples extracted in homogenization buffer. A 50 µL aliquot of the extract was mixed with 450 µL of 0.09% laminarin solution and incubated at 37°C for 60 min. The enzymatic reaction was stopped by adding 500 µL of dinitrosalicylic acid reagent, followed by boiling the mixture at 100°C for 10 min. After cooling on ice for 5 min, the reaction mixture was diluted to a final volume of 5 mL using distilled water. Absorbance was measured at 550 nm using the spectrophotometer. The enzyme activity was calculated based on the method of Vázquez-Garcidueñas et al. (1998) using Equation 5:

$$\beta\text{-1,3-Glucanase activity} = (0.0002 \times \text{Absorbance}_{550}) - 0.0886 \qquad (5)$$

The total protein content was determined based on the method of Bradford (1976) and calculated using Equation 6:

$$\text{Total protein} = (0.0015 \times \text{Absorbance}_{595}) - 0.0389 \qquad (6)$$

The IAA accumulation was determined in the harvested Napier grass root tips that had been frozen in liquid nitrogen and then ground using a mortar and pestle. A 0.1 g portion of the ground frozen tissue was homogenized in 1 mL of extraction buffer and centrifuged at 8,000 revolutions per minute for 15 min. The resulting supernatant (1 mL) was mixed with 2 mL of Salkowski's reagent and incubated in the dark at room temperature for 30 min. Absorbance was measured at 550 nm using the spectrophotometer. The IAA content was quantified by comparing the absorbance values to a standard curve and calculated using the equation provided by Baker and Hargreaves (2000) and Leveau and Lindow (2005):

$$\text{IAA content} = (0.0157 \times \text{Absorbance}_{550}) - 0.0043$$

Experimental design and statistical analysis

The experiment was conducted using a completely randomized design. Data were analyzed using one-way analysis of variance (ANOVA), and treatment means were separated using Duncan's multiple range test (DMRT) at $p < 0.05$. Results are presented as mean ± SD.

Ethics statements

Ethical approval was not required for this study as it did not involve human or animal subjects.

Results

Co-culturing *Bacillus* strains and *T. asperellum* at varying concentrations influenced their colony growth patterns. At low concentrations (1.75×10^6 CFU/mL *Bacillus* and 1×10^4 spores/mL *T. asperellum*; T1), *T. asperellum* produced full colony growth (8.70 ± 0.00 cm), while *Bacillus* growth was limited (1.63 ± 1.55 cm), indicating weak antagonism. At higher concentrations (3.5×10^6 CFU/mL *Bacillus* and 1×10^8 spores/mL *T. asperellum*; T4), both organisms produced reduced colony diameters, with *T. asperellum* at 6.74 ± 1.65 cm and *Bacillus* at 3.04 ± 1.47 cm, suggesting increased competitive interactions. Notably, in T2, *T. asperellum* produced slightly enhanced growth (8.27 ± 0.93 cm) with 1.75 × 10^6 CFU/mL *Bacillus,* implying a potential stimulatory effect at this concentration. Chitinase activity was detected only at highest inoculum levels (T4), whereas cellulase activity was not detected at any inoculum concentration. These findings indicated a shift from neutral or slightly beneficial interactions to competitive inhibition as microbial concentrations increased (Table 1). Under single-culture conditions, neither *Bacillus* nor *T. asperellum* alone produced detectable chitinase activity within the 5 d of incubation on the assay medium. In contrast, chitinase activity was observed in the high-concentration co-culture treatments, suggesting enzyme induction triggered by microbial interaction. These enzymes were measured because they are important indicators of the biocontrol potential of *Bacillus* spp. and *T. asperellum*. Although their primary function is in pathogen suppression (Rao et al., 2016), the resulting reduction of pathogen pressure can indirectly promote plant growth.

In greenhouse trial I at age 5 mth, there was a significant response in the plant growth in both the Pak Chong 1 and Taiwan Napier grass cultivars. In Pak Chong 1, *Bacillus* applied at 14 d intervals × 2 times produced the highest plant height (64.64% at 125.20 ± 26.30 cm), while in Taiwan, the highest (42.22%) was obtained with *Bacillus* applied at 7 d intervals × 4 times. Stem diameter improved the most with *Bacillus* applied at 14-day intervals for Pak Chong 1 (15.85%) and *T. asperellum* for Taiwan (16.32%). Although the overall changes were modest, these treatments outperformed the control in both cultivars. In Pak Chong 1, the largest increase in leaf number (277.55%) was obtained with *Bacillus* applied at 14 d intervals × 2 times. In Taiwan, the highest leaf number (209.57%) was recorded with the combined treatment of *T. asperellum* at 14 d intervals × 2 times. These results highlighted the cultivar-specific responses to microbial application schedules, with *Bacillus* showing stronger growth-promoting effects, particularly on foliage production (Fig. 1).

There was only slight variation in the macronutrient contents (N, P, K) among the different microbial treatments, suggesting that the treatments had limited or no consistent effect on nutrient accumulation in Napier grass. In Pak Chong 1, total N ranged narrowly from 0.89% to 1.12%, P from 0.29% to 0.33% and K from 3.33% to 3.55% across treatments. Similarly, in Taiwan, slight increases in N and K were observed in some treatments (1.45% N with *Bacillus* and 4.59% K with *T. asperellum*), these differences were small and did not indicate a strong or consistent treatment effect. The postharvest data provided additional insight into nutrient redistribution. In Pak Chong 1, nutrient levels in postharvest leaves (0.44% N, 0.10% P, 1.64% K) were higher in nitrogen but lower in phosphorus and potassium compared to postharvest stems (0.22% N, 0.18% P, 1.86% K), indicating differential nutrient partitioning between tissues. After water pressing, the N content declined further (0.20%), but the P and K concentrations increased (0.27% and 3.45%, respectively), likely due to moisture loss concentrating solutes, especially potassium (Table 2).

Table 1 Colony growth (colony diameter and distance between colonies) of mixed *Bacillus* strains and *Trichoderma asperellum* in co-culture on potato dextrose agar after 5 d incubation at room temperature. Percent inhibition versus the control is reported, and chitinase and cellulase activities are recorded qualitatively (ND, −, +).

	Microbe	Concentration	Colony diameter (cm)	Distance between colonies (cm)	% Inhibition versus control	Chitinase	Cellulase
1	*Trichoderma*	10^4 spores/mL	8.70 ± 0.00^{a}	0.36 ± 0.38	0	ND	ND
	Bacillus	1.75 × 10^6 CFU/mL	1.63 ± 1.55^{de}		49.22	ND	ND
2	*Trichoderma*	10^8 spores/mL	8.27 ± 0.93^{abc}	0.33 ± 0.20	-3.38	ND	ND
	Bacillus	1.75 × 10^6 CFU/mL	2.13 ± 2.03^{de}		33.64	ND	ND
3	*Trichoderma*	10^4 spores/mL	8.70 ± 0.00^{a}	0.14 ± 0.18	0	ND	ND
	Bacillus	3.5 × 10^6 CFU/mL	1.77 ± 2.53^{d}		46.36	ND	ND
4	*Trichoderma*	10^8 spores/mL	6.74 ± 1.65^{abc}	0.33 ± 0.18	15.75	+	–
	Bacillus	3.5 × 10^6 CFU/mL	3.04 ± 1.47^{d}		7.88	+	–
5	*Trichoderma*	10^4 spores/mL	8.70 ± 0.00^{a}		0	ND	ND
6	*Trichoderma*	10^8 spores/mL	8.00 ± 0.72^{ab}		0	–	–
7	*Bacillus*	1.75 × 10^6 CFU/mL	3.21 ± 2.15^{def}		0	ND	ND
8	*Bacillus*	3.5 × 10^6 CFU/mL	3.30 ± 0.69^{d}		0	–	–

Mean ± SD values from replicated measurements of colony diameter after 5 d incubation.

% inhibition versus control was calculated for each microorganism relative to its corresponding single-culture control at the same inoculum concentration.

Presence of a clear zone (+) around colonies indicates enzymatic activity; absence (–) indicates no activity and ND = not detected.

Different lowercase letters within a column indicate significant differences according to Duncan's multiple range test ($p < 0.05$).

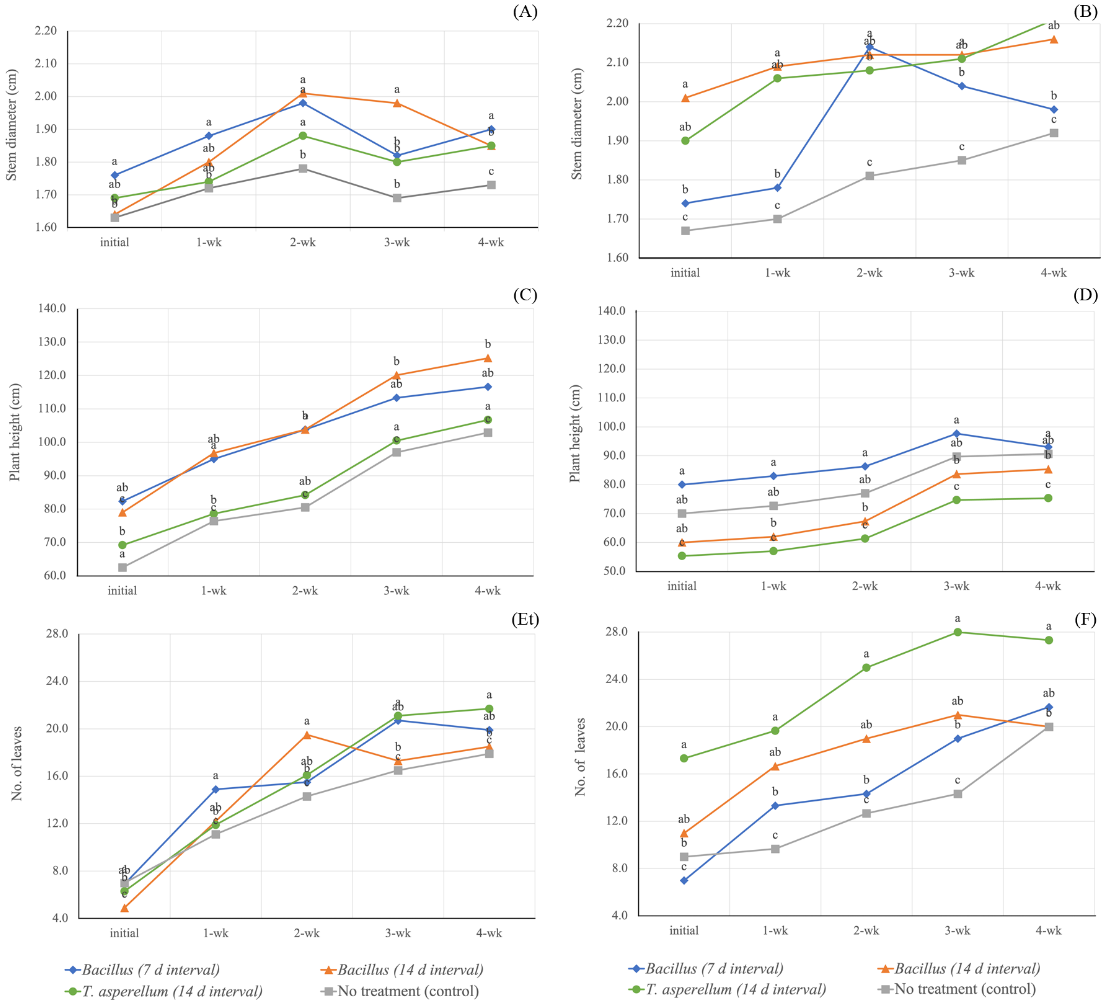

Fig. 1 Growth responses of Napier grass cultivar Pak Chong 1 (A), (C), (E) and Taiwan (B), (D), (F) to *Bacillus* spp. and *Trichoderma asperellum* treatments under greenhouse conditions. Stem diameter (A), (B); plant height (C), (D); number of leaves (E), (F). Measurements were taken on plants at age 5 mth of plant age before treatment and weekly for 4 wk after treatment. Different lowercase letters at each time point indicate significant differences among treatments according to Duncan's multiple range test ($p < 0.05$).

Table 2 Total nitrogen (N), phosphorus (P) and potassium (K) contents (%) of Napier grass cultivars Pak Chong 1 and Taiwan after postharvest processing and microbial treatments (*Bacillus* spp. or *Trichoderma asperellum*) applied at the indicated intervals.

Treatment description	Total N (%)	Total P (%)	Total K (%)
Napier grass Pak Chong 1			
Postharvest leaves	0.44 ± 0.04	0.1 ± 0.01	1.64 ± 0.10
Postharvest stem	0.22 ± 0.02	0.18 ± 0.02	1.86 ± 0.17
After water pressing out	0.2 ± 0.02	0.27 ± 0.03	3.45 ± 0.21
Bacillus (7 d interval)	1.05 ± 0.09	0.32 ± 0.02	3.38 ± 0.28
Bacillus (14 d interval)	0.89 ± 0.08	0.29 ± 0.03	3.33 ± 0.32
T. asperellum (14 d interval)	1.03 ± 0.09	0.33 ± 0.03	3.53 ± 0.30
No treatment (control)	1.12 ± 0.10	0.32 ± 0.03	3.55 ± 0.30
Napier grass Taiwan			
Bacillus (7 d interval)	1.27 ± 0.13	0.29 ± 0.03	4.24 ± 0.32
Bacillus (14 d interval)	1.45 ± 0.11	0.41 ± 0.02	4.52 ± 0.37
T. asperellum (14 d interval)	1.26 ± 0.13	0.41 ± 0.04	4.59 ± 0.46
No treatment (control)	1.01 ± 0.07	0.36 ± 0.03	4.34 ± 0.43

Data are presented as mean ± SD values.

Based on the results regarding the growth and physiological responses for the Pak Chong 1 Napier grass aged 1 mth to microbial treatments in the greenhouse trial II, *Bacillus* (14 d interval × 2) treatment led to the highest increase in plant height (134.5%), followed by *T. asperellum* (94.0%), while the control had the lowest increase (64.1%). Although the control group had the greatest relative increase in stem diameter (35.4%), differences in stem diameter and number of leaves among treatments were not significantly different. Similarly, leaf number increased modestly across all treatments; however, the differences were not significant. The chlorophyll content (SPAD value) at 2 mth tended to be higher in the *Bacillus*-treated plants, while at 3 mth, *T. asperellum* had the highest SPAD reading. However, these differences were not significantly different and so should be interpreted cautiously. For Taiwan, *Bacillus* again promoted the greatest height increase (96.9%), while *T. asperellum* had a limited effect (25.7%) and the control group was moderately impacted (38.5%). Similar to Pak Chong 1, stem diameter and leaf number in Taiwan did not differ significantly among treatments. Chlorophyll content increased over time in all treatments, with the control showing the highest SPAD value at 3 mth (83.72%). This trend may reflect natural plant development or late-stage greening rather than a treatment effect. Based on these results, it seemed that *Bacillus* improved early shoot elongation and chlorophyll content, particularly in Pak Chong 1, while *T. asperellum* had weaker or more variable effects and responses were cultivar dependent. (Figs. 2 and 3).

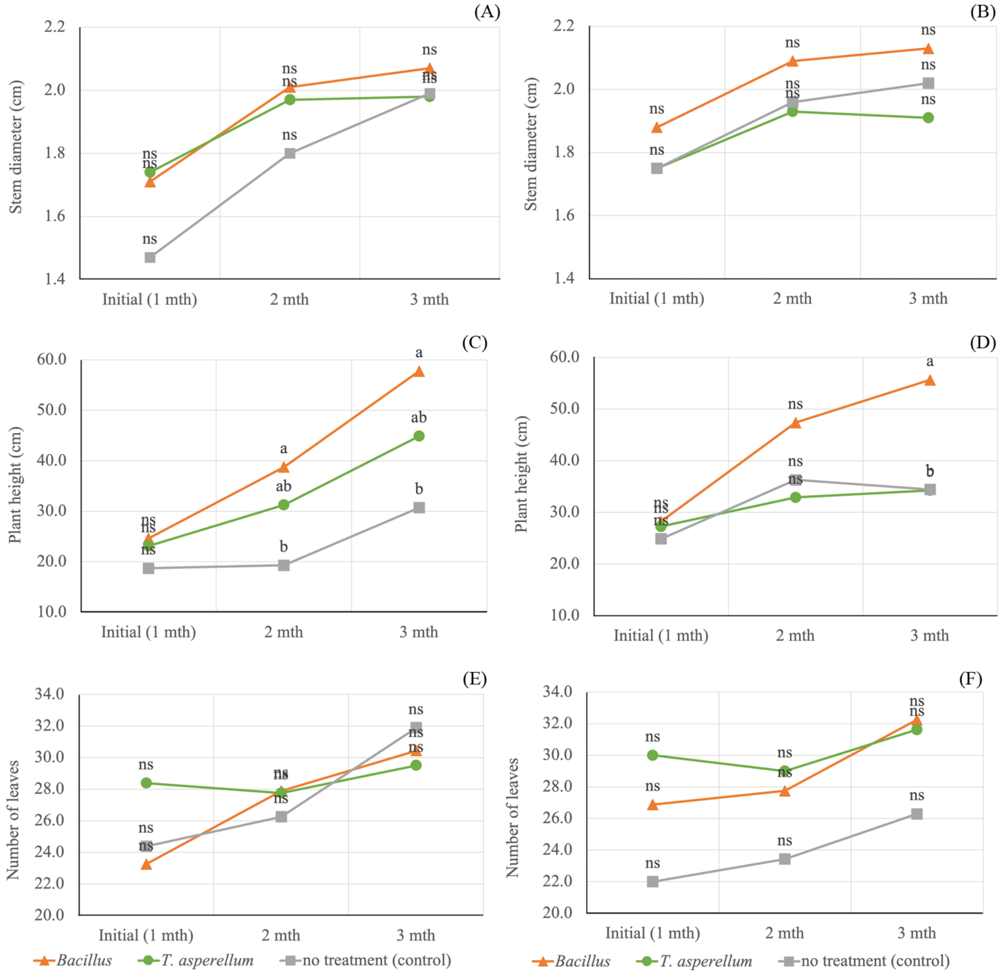

Fig. 2 Growth responses of Napier grass cultivars Pak Chong 1 (A), (C), (E) and Taiwan (B), (D), (F) to *Bacillus* spp. and *Trichoderma asperellum* treatments under greenhouse conditions. Stem diameter (A), (B); plant height (C), (D); number of leaves (E), (F) were measured at 1, 2 and 3 mth after treatment. Different lowercase letters indicate significant differences among treatments according to Duncan's multiple range test ($p < 0.05$); ns = non-significant differences.

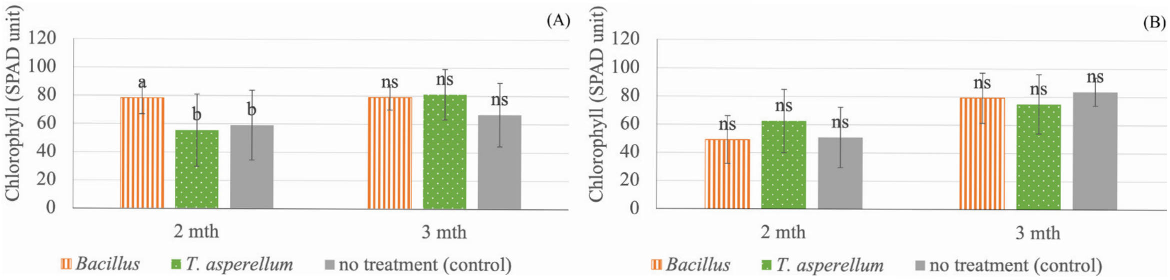

Fig. 3 Chlorophyll content (SPAD units) of Napier grass cultivars Pak Chong 1 (A) and Taiwan (B) under *Bacillus* spp. and *Trichoderma asperellum* treatments in greenhouse conditions. Measurements were taken at 2 and 3 mth after treatment. Different lowercase letters indicate significant differences among treatments according to Duncan's multiple range test ($p < 0.05$); ns = non-significant differences.

Regarding biomass production, the microbial treatments applied to the Napier grass following affected fresh weight more than dry weight, with cultivar-specific differences. In Pak Chong 1, there were no significant differences in either the fresh or dry biomass. Although *Bacillus* (14 d interval × 2) led to the highest fresh stem weight (244.45 ± 94.94 g) and leaf weight (100.57 ± 22.49 g), the variation among treatments was not significant. Dry weight values followed a similar trend but again, there were no significant differences across treatments. In contrast, there were significant differences in fresh biomass for the Taiwan cultivar, where the *Bacillus*-treated plants produced the highest fresh stem (290.61 ± 16.72 g) and leaf (123.42 ± 14.55 g) weights, with both being significantly greater than for the control. *T. asperellum* had intermediate values and did not differ significantly from *Bacillus*. However, for both cultivars, the dry weights of the stem and leaves were not significantly different among treatments, suggesting that microbial inoculation may enhance water content or succulence rather than structural biomass accumulation (Fig. 4). Increases in fresh biomass were observed in *Bacillus*-treated Taiwan; however, the dry weight differences were not significant, suggesting that microbial inoculation may affect the water content rather than structural biomass accumulation.

The activity of defense-related enzymes and the IAA content in the Napier grass cultivars treated with microbial inoculants, varied between treatments and cultivars. In Pak Chong 1, the *T. asperellum* treatment markedly enhanced all enzyme activities of PAL (5.85 µg trans-cinnamic acid/min·mg protein), POX (0.22 ΔAbs/min·mg protein) and β-1,3-glucanase (86.03 µg glucose/min), compared to the *Bacillus* and control treatments. *Bacillus* moderately increased enzyme activities over the control, particularly for β-1,3-glucanase (55.42 µg glucose/min). Notably, the IAA content was relatively similar among treatments (10.96–12.18 µg/g). For the Taiwan cultivar, *T. asperellum* again induced high PAL (5.26) and β-1,3-glucanase (72.65) activity, while the control unexpectedly showed comparable PAL (5.63) and POX (0.20) activity levels but with a lower IAA content (3.44 µg/g). Notably, the *T. asperellum* treatment had the highest IAA content (12.64 µg/g), suggesting a cultivar-specific hormonal response (Table 3).

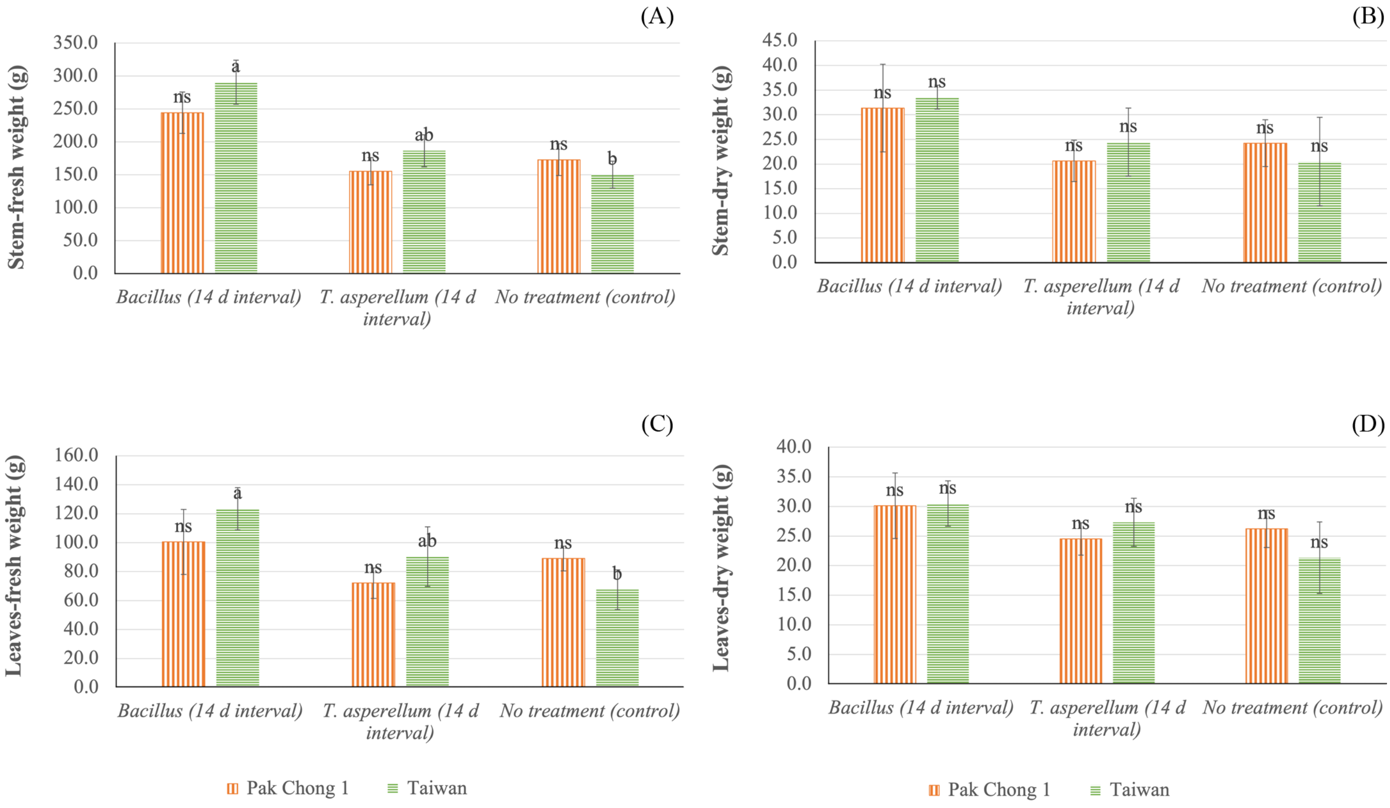

Fig. 4 Effects of *Bacillus* spp. and *Trichoderma asperellum* treatments on fresh weight (A), (C) and dry weight (B), (D) of Napier grass cultivars Pak Chong 1 and Taiwan at 3 mth of growth. Bars represent mean values (n = 8) and error bars indicate ± SD. Different lowercase letters indicate significant differences among treatments within each cultivar according to Duncan's multiple range test ($p < 0.05$); ns = non-significant differences.

Table 3 Activity of defense-related enzymes (PAL, POX, and β-1,3-glucanase) and indole-3-acetic acid (IAA) contents in Napier grass cultivars Pak Chong 1 (PC) and Taiwan (TW) following *Bacillus* spp. or *Trichoderma asperellum* treatments under greenhouse conditions.

Cultivar	Treatment	PAL (µg trans-cinnamic acid released/min·mg protein)	POX (ΔAbs at 470 nm/ min·mg protein)	β-1,3-Glucanase (µg glucose released/min)	IAA (µg/g)
PC	*Bacillus* (14 d interval)	3.93 ± 0.060^{ab}	0.14 ± 0.006^{b}	55.42 ±0.025^{ab}	11.99 ± 0.995^{a}
	T. asperellum (14 d interval)	5.85 ± 0.025^{a}	0.22 ± 0.013^{a}	86.03 ± 0.035^{a}	10.96 ± 0.175^{a}
	No treatment (control)	2.57 ±0.195^{b}	0.01 ± 0.001^{c}	31.68 ± 0.100^{b}	12.18 ± 0.035^{a}
TW	*Bacillus* (14 d interval)	2.86 ± 0.06^{b}	0.01 ± 0.001^{c}	27.05 ± 0.495^{b}	9.00 ± 0.510^{a}
	T. asperellum (14 d interval)	5.26 ± 0.250^{a}	0.18 ± 0.004^{a}	72.65 ± 0.200^{a}	12.64 ± 0.050^{a}
	No treatment (control)	5.63 ± 0.180^{a}	0.20 ± 0.002^{a}	58.75 ± 0.175^{ab}	3.44 ± 0.200^{b}

Mean ± SD values within a column followed by different lowercase letters indicate significant differences ($p < 0.05$) among treatments across cultivars according to Duncan's multiple range test ($p \geq 0.05$).
PAL= phenylalanine ammonia-lyase; POX = peroxidase.

Discussion

Based on the current results, there were significant effects from microbial inoculation with mixed *Bacillus* strains and *T. asperellum* on the growth, physiology and nutrient status of the Napier grass cultivars Pak Chong 1 and Taiwan under greenhouse conditions. The improvements in growth parameters, nutrient accumulation, chlorophyll content, biomass, enzyme activity and IAA content were closely linked to microbial concentration and application frequency.

Regarding microbial interaction and compatibility, co-culturing *Bacillus* strains and *T. asperellum* revealed dynamic interactions influenced by microbial density. At lower concentrations (3.5 g/L *Bacillus,* 1 × 10^4 spores/mL *T. asperellum*), colony diameters remained high for *T. asperellum* (≥ 8.70 cm), while *Bacillus* growth was suppressed, indicating a competitive interaction. In contrast, higher concentrations (7.0 g/L *Bacillus*, 1 × 10^8 spores/mL *T. asperellum*) led to reductions in colony size for both microbes, with chitinase detected, suggesting partial compatibility despite competitive effects. These findings aligned with other research on microbial interaction dynamics (Karuppiah et al., 2019a; Karuppiah et al., 2019b; Senkovs et al., 2021; Hao et al., 2024). The detection of chitinase activity only in high-concentration co-cultures may indicate induction of enzyme production under competitive conditions. Similar interaction-induced chitinase production has been reported when antagonistic microorganisms are co-cultured, likely as a defensive or offensive strategy against competing species. It is crucial to identify the optimal concentrations for co-application to harness synergistic benefits while avoiding competitive suppression (Harman, 2006; Vinale et al., 2008). These enzymes were measured because they are important indicators of the biocontrol potential of *Bacillus* spp. and *T. asperellum* (Ramada et al., 2010; Kaur et al., 2020; Sahouli et al., 2020). Although their primary function is in pathogen suppression, the resulting reduction of pathogen pressure could indirectly promote plant growth. Furthermore, cellulase can facilitate root colonization by biocontrol agents, thereby improving nutrient uptake efficiency. Including these assays allowed for better characterization of the functional traits of the bioagents beyond direct growth promotion (Nawrocka and Małolepsza, 2013; Adedayo and Babalola, 2023).

The dual culture assay indicated a competitive rather than synergistic interaction between *Bacillus* spp. and *T. asperellum* under *in vitro* conditions, as reduced colony growth was observed for both organisms compared to their single cultures. Nevertheless, both were selected for greenhouse trials due to their complementary plant growth-promoting traits. The bioagents were applied separately in each treatment to avoid potential antagonism and to assess their independent effects. Under these conditions, both *Bacillus* spp. and *T. asperellum* effectively enhanced plant growth and physiological responses in two Napier grass cultivars (Pak Chong 1 and Taiwan). While the *in vitro* co-culture assay revealed no significant difference in colony growth inhibition between the low and high concentrations of either bioagent, the higher concentration (1 × 10^8 spores/mL for *T. asperellum* and 3.5 × 10^6 CFU/mL for *Bacillus*) was selected for the greenhouse experiments to compensate for potential microbial population decline under environmental conditions. Using the higher dose ensured adequate colonization and persistence in the rhizosphere.

Microbial treatments significantly influenced growth traits in Napier grass, with cultivar-dependent responses. In Pak Chong 1, *Bacillus* applied at 14 d intervals produced the greatest plant height and leaf number, while in Taiwan, the same treatment resulted in the highest relative height gain. *T. asperellum* also enhanced shoot traits, particularly leaf production, though its effects were generally more moderate.

These responses reflected the differential sensitivities of cultivars to microbial inoculation schedules. The observed growth improvements aligned with other studies demonstrating the biostimulant roles of *Trichoderma* and *Bacillus*. For example, Pelagio-Flores et al. (2017) highlighted that *Trichoderma*-induced rhizosphere acidification altered auxin distribution, influencing root development and, by extension, shoot architecture. Fiorentino et al. (2018) further demonstrated that *Trichoderma*-based biostimulants enhanced stem and leaf biomass by improving nitrogen uptake efficiency. Zhu et al. (2024) and Yang et al. (2024b) reported that co-application of *Bacillus* and *Trichoderma* modulated plant defense responses, reduced disease severity and supported shoot health through microbial synergy and stress mitigation. In the current study, despite clear growth responses, the nutrient content (N, P, K) varied only slightly among treatments, suggesting that the primary effect was not enhanced nutrient uptake but rather microbial signaling or hormonal modulation. In addition, the postharvest data showed differential nutrient partitioning, with nitrogen enriched in leaves and phosphorus and potassium being more concentrated in stems. Notably, increased P and K levels following water pressing, likely resulting from moisture loss and solute concentration. These findings supported the conclusion that *Bacillus* and *T. asperellum* promoted shoot development primarily through physiological and biochemical stimulation rather than nutrient enrichment.

The tested microbial treatments influenced the growth traits in both Napier grass cultivars at age 5 mth, with distinct cultivar-specific responses. In Pak Chong 1, *Bacillus* applied at 14 d intervals resulted in the greatest absolute plant height, while *T. asperellum* showed moderate improvement. In Taiwan, the same *Bacillus* treatment also produced the highest relative height gain. Stem diameter increased most with *Bacillus* in Pak Chong 1 and with *T. asperellum* in Taiwan, suggesting differences in cultivar responsiveness. Leaf number was most responsive to microbial application, especially with *Bacillus* at 14 d intervals in Pak Chong 1 and 7 d intervals in Taiwan. In addition, *T. asperellum* enhanced leaf production substantially. The reference provided by Pelagio-Flores et al. (2017) explained the influence of *Trichoderma* on root growth, which can be extrapolated to its effects on stem and leaf development as well. The current study highlighted that *Trichoderma*-induced acidification mediated interactions between the fungus and plants, significantly impacting root architecture, which was closely tied to overall plant health and growth, including stem and leaves. As the research demonstrated, acidification led to auxin redistribution, affecting growth patterns that can be crucial for leaf expansion and health. Furthermore, the detrimental effects noted in root growth and subsequent leaf pigmentation provided insight into how fungal interactions can influence stem and leaf development through stress responses. Zhu et al. (2024) discussed how the application of PGPR, specifically *Bacillus subtilis* and *Bacillus licheniformis*, in conjunction with *T. longibrachiatum*, influenced the physiological responses of common vetch (*Vicia sativa*) against anthracnose pathogens. This aligned with the task of exploring how *Trichoderma* and *Bacillus* enhanced plant growth by altering the microbial composition and promoting plant defense mechanisms. The current study highlighted that both *Bacillus* and *Trichoderma* acted effectively as biocontrol agents, reducing disease severity and fostering beneficial microbial interactions, which ultimately contributed to improved plant health and productivity. Yang et al. (2024b) elucidated the role of *Bacillus* sp. in inhibiting the harmful effects of *Trichoderma* spp. on White *Hypsizygus marmoreus*, highlighting its potential to enhance the nutritional quality and shelf life of the mushroom. Their findings indicated that *Bacillus* sp. produces enzymes that directly target *Trichoderma*, along with a synergistic effect when combined with salicylic acid, indicating that *Bacillus* sp. contributes to maintaining a healthy stem and leaves through protective mechanisms against fungal infections, ultimately supporting the assertion that it enhances overall plant quality and resilience. Fiorentino et al. (2018) provided evidence of how *Trichoderma*-based biostimulants enhanced crop yields, specifically for leafy vegetables such as lettuce and rocket, by improving nitrogen uptake efficiency and nutritional quality. Other studies demonstrated that *Trichoderma* strains enhanced plant growth under varying nitrogen levels, particularly under low-nitrogen conditions, by modifying rhizosphere composition and pH, thereby promoting stem and leaf development (Sharma et al., 2020; Amanullah and Khan, 2023). In the current study, macronutrient contents (N, P, K) varied slightly across treatments, indicating minimal effects on nutrient accumulation. The postharvest data revealed a higher nitrogen content in the leaves and higher phosphorus and potassium contents in the stems with increased P and K after water pressing, likely due to moisture loss. Overall, the microbial treatments, particularly *Bacillus,* promoted shoot growth more than nutrient uptake, highlighting the role of microbial signals in growth enhancement.

The current experiment assessed the effects of microbial inoculants on Napier grass aged 1 mth under greenhouse conditions, revealing cultivar-specific differences in growth, physiological responses and biomass accumulation. In Pak Chong 1, *Bacillus* (14-d interval) enhanced plant height and the chlorophyll content at 2 mth, indicating strong early stimulation of shoot elongation and photosynthetic efficiency. Although the stem diameter and leaf number did not differ significantly, the trends suggested modest improvement. *T. asperellum* maintained the highest SPAD value at 3 mth, reflecting sustained physiological benefits beyond the initial growth phase. In Taiwan, *Bacillus* again promoted height gain, while other parameters were less responsive. Notably, the SPAD values increased over time in all treatments, with the control group peaking at 3 mth, likely due to delayed greening or natural recovery. Biomass accumulation further supported these differential responses. In Pak Chong 1, the microbial treatments modestly increased both the fresh and dry weights though not significantly. In contrast, *Bacillus* significantly improved the fresh stem and leaf weights in Taiwan, suggesting enhanced water retention or tissue succulence rather than true structural biomass gains. This aligned with the typical early-stage physiological effects of microbial priming, where the water content and metabolic activity increase before any notable dry matter accumulation. These findings were consistent with earlier reports. For example, *T. harzianum* and related isolates have been shown to promote seedling growth, biomass and nutrient uptake in several crops (Santos et al., 2019; Mahmoodian et al., 2022). Similarly, *T. longibrachiatum* enhanced the chlorophyll content, fresh weight and physiological resilience in *Pinus massoniana* seedlings under stress (Yu et al., 2023). These effects were likely mediated through improved root architecture, hormonal signaling and rhizosphere conditioning.

Enzymatic and hormonal profiles confirmed the role of the microbial treatments in modulating plant defense and development. In both cultivars, *T. asperellum* significantly enhanced the activity of defense-related enzymes (PAL, POX and β-1,3-glucanase), with more pronounced effects in Pak Chong 1. These results were consistent with other reports highlighting *Trichoderma*'s capacity to induce systemic resistance and activate defense pathways (Yedidia et al., 1999; Harman et al., 2006). Notably, *T. asperellum* elevated the IAA contents in Taiwan, suggesting cultivar-dependent hormonal modulation under microbial influence. In contrast, the *Bacillus* promoted moderate enzyme activity and consistently induced IAA accumulation, particularly in Pak Chong 1 (12.16 μg/g fresh weight), aligning with its known role in root development and growth regulation (Tsotetsi et al., 2022). These findings suggested that *T. asperellum* primarily enhanced plant defense, while *Bacillus* supported a balance between growth promotion and defense priming. Beyond biochemical responses, these microbial treatments also hold broader implications for sustainable agriculture. Both microbial agents can improve soil fertility and plant health through mechanisms such as phosphate solubilization, nitrogen fixation, IAA synthesis and pathogen suppression (Stein, 2005; Idris et al., 2007; Spaepen et al., 2007; Liang et al., 2024). In the current study, POX activity was assessed because this enzyme plays a central role in plant defense responses, particularly by strengthening cell walls through lignin deposition and by contributing to the generation of reactive oxygen species that inhibit pathogen growth (Chagas et al., 2008). POX activity was strongly induced by *T. asperellum* in both cultivars, reaching 0.22 ΔAbs/min/mg protein in Pak Chong 1 and 0.18 ΔAbs/min/mg protein in Taiwan, compared with negligible activity in their respective controls. In addition, *Bacillus* elevated POX in Pak Chong 1, though at a lower level, while in Taiwan its effect was minimal. These results suggested that *T. asperellum* was more effective than *Bacillus* in activating oxidative defense pathways and that cultivar background influenced the magnitude of POX induction.

These results supported the integration of microbial bioproducts as sustainable tools in forage production and integrated nutrient management systems. However, the findings were based on controlled greenhouse conditions. Field trials across different agroecological zones are necessary to validate practical applications. In addition, future research should examine microbial persistence in the soil, the long-term effects on forage yield and quality, as well as cost-effectiveness for large-scale use.

Conclusion

The microbial bioproducts, particularly mixed *Bacillus* strains, significantly enhanced the growth, physiological performance and nutrient uptake of Napier grass under greenhouse conditions. Repeated application of *Bacillus* promoted early shoot development, increased stem diameter, leaf number, chlorophyll content, fresh biomass and IAA accumulation especially in the Taiwan cultivar. In contrast, *T. asperellum* was more effective in inducing defense-related enzyme activities (PAL, POX and β-1,3-glucanase), contributing to enhanced physiological resilience. Overall, cultivar-specific responses were evident, with Taiwan showing greater sensitivity to microbial treatments than Pak Chong 1. These findings highlighted the potential of microbial inoculants as sustainable strategies for improving Napier grass productivity and plant health, with promising applications in forage production and bioenergy development.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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