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ARTICLE

Biopriming salt-tolerant microbial isolates to chilli and pak choy seeds: a study on salinity tolerance and physiological responses of treated seeds

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ABSTRACT *Trichoderma asperellum* and *Pseudomonas fluorescens* were first established for their tolerance to salinity. They were bioprimed onto two common vegetable seeds (pak choy and chilli) using sodium alginate (for *T. asperellum*, TAB) and xanthan gum (for *P. fluorescens*, PFB) and sown into soils amended with NaCl (salinity stress). Both *T. asperellum* and *P. fluorescens* have high salt tolerance (up to 250 mM of NaCl) concentration). Bioprimed seedlings had sustained growth in saline soils (2.72 – 3.05 dS/m). Pak choy seedlings benefited the most from biopriming with TAB or PFB seedlings, showing enhanced fresh weight, shoot length, root length and germination, compared to non-bioprimed seedlings. For chilli seedlings, only shoot length was enhanced. Tolerance to salinity was marked by lower levels of proline (0.62-2.73 µmol/g fr. wt.), total phenolic content (80.29-130.10 mg GAE/100 g fr. wt.), and malondialdehyde (0.29-0.61 µmol/g fr. wt.) compared to non-bioprimed seedlings (NB) under salinity stress (1.87-3.55 µmol/g fr. wt. for proline, 105.60-278.82 mg GAE/100 g fr. wt. for total phenolic content, 0.33-0.61 µmol/g fr. wt. for malondialdehyde). Our early observations showed the potential of biopriming salt-tolerant isolates to enhance survival of important vegetable crops in saline soils.

KEY WORDS
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Pseudomonas fluorescens
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Introduction

Salinity stress is one of the most prevalent limiting abiotic factors to crop growth. Salt concentrations in soils increase as a consequence of weathering processes, or anthropogenic factors (Etesami and Beattie 2017). It affects approximately 20% of agricultural land and reduces 10% of cultivated land annually, thereby impacting crop production worldwide (Tuteja et al. 2012). Soils with high salinity stress are caused by poor drainage or that they have high salt deposition from irrigation. As such, soils with salinity stress are more commonly found in the coastal regions, and arid or semiarid regions. Excessive salt accumulation in the soil affects soil aggregation, productivity, and fertility, leading to poor crop productivity. They interfere with plant growth as the water potential in plant tissues are lowered, leading to higher intake of cationic inorganic salts that cause toxicity to plants (Tuteja et al. 2012; Biswas and Biswas 2018).

In this study, the vegetable crops studied are pak choy (Chinese cabbage; *Brassica rapa* subsp. *chinensis*) and chilli (*Capsicum annuum*). Pak choy and chilli are important vegetable crops, generating an average of 145,427 Mt (RM 225 million) and 29,568 Mt (RM 282 million) production in 2019, respectively (FAOSTAT 2019). However, with severe ion toxicity and salinity stress, the vegetative growth and production of pak choy can be adversely affected (Jan et al. 2016). Chillies are equally impacted by salinity stress, as the low water potential in soils affect nutrient uptake that are important for seedling emergence and fruit production (Howlader et al. 2018; Loganayaki et al. 2020). To manage salinity stress, the strategy is to alleviate the toxicity of salt ions in plants so that improved crop growth can be attained. To achieve this, this study adopts the biological approach in which salt-tolerant microorganisms are explored to confer improved plant growth under salinity stress. This approach also provides a more sustainable method to increase agricultural productivity.

To enhance the delivery of salt-tolerant isolates, seed biopriming (or coating) is introduced. Seed coating (biopriming) is carried out using biopolymers to coat the microbial inoculants (salt-tolerant microorganisms) onto seeds prior to exposure to salinity stress (Ma 2019). This is a promising technique to protect seeds from salinity stress (Singh et al. 2016a; Ma 2019). Seed biopriming with salt-tolerant microorganisms has been reported for several crops such as wheat (Safari et al. 2018; Dief et

al. 2021), maize (Pehlivan et al. 2017; Singh et al. 2019), soybean (Khomari et al. 2018), French bean (Gupta and Pandey 2019) and okra (Habib et al. 2016). However, it has not been attempted for a leafy vegetable crop like pak choy (*B. rapa* subsp. *chinensis*) nor on chilli (*C. annuum*). The salt-tolerant microorganisms used can be sourced from endophytic fungi, biocontrol agents (BCA) or plant growth-promoting microorganisms. These beneficial microorganisms are ideal as they demonstrate bioactivities as well as promote plant growth even in the presence of salinity stress. These microorganisms render tolerance against salinity stress by promoting seed emergence, growth and by protecting seedlings from salt toxicity via osmolyte accumulation (proline, soluble polysaccharides), antioxidant production and reduction in malondialdehyde (MDA) lipid peroxidation in plants (Dief et al. 2021).

In this study, the biopolymers sodium alginate and xanthan gum were selected as coating agents to coat the selected beneficial isolates (*Trichoderma asperellum* and *Pseudomonas fluorescens*) to chilli and pak choy seeds (Chin et al. 2021, 2022a, 2022b). Our earlier studies have established the beneficial characteristics of both *T. asperellum* and *P. fluorescens* and optimized their biopriming conditions (Chin et al. 2021, 2022a, 2022b). The salt-tolerant profile of *T. asperellum* and *P. fluorescens* was first assessed, followed by biopriming of seeds. The efficacy of salt-tolerant isolates in rendering beneficial effect was determined by the changes in biochemical markers (proline, total phenolic content and malondialdehyde levels) and the vegetative growth parameters of the seedlings. This study aims to reveal the potential of biopriming salt-tolerant isolates to seeds and their subsequent efficacy in improving salinity tolerance in the bioprimed seedlings.

Materials and Methods

Culture preparation

Trichoderma asperellum T2 (GenBank accession No. KT964564) was obtained from stock cultures and maintained on potato dextrose agar (PDA, Merck, Germany) at room temperature (25 ± 2 °C) (Ting and Jioe 2016; Cheong et al. 2017). Spores were harvested from a 5-day-old culture of *T. asperellum* cultured on PDA. The spore suspension was obtained by dispensing 10 mL of sterile distilled water to the cultures, with the spores gently dislodged using a glass spreader. The suspension was collected and added with 0.05% v:v Tween 20 (surfactant) (Merck, Germany) for better spore distribution, and adjusted to 10^9 spores/mL using a haemocytometer. For *Pseudomonas fluorescens*, the culture was established from glycerol stock and maintained on King's B agar (Fluka, Switzerland) at room temperature (25 ± 2 °C). To prepare cell suspensions, *P. fluorescens* was first cultured in nutrient broth (Merck, Germany) and incubated overnight. The culture was then adjusted to OD_{625} of 0.1 (approximately 10^8 cells/mL) before use.

Establishing salinity-tolerance profile of isolates

T. asperellum and *P. fluorescens* were evaluated for their tolerance against salinity stress. *T. asperellum* was inoculated centrally onto PDA plates supplemented with varying concentrations of sodium chloride (J. Kollin, UK) (0, 50, 100, 150, 200 and 250 mM of NaCl) and incubated at 25 ± 2 °C for 7 days. The growth and macroscopic characteristics of *T. asperellum* on PDA were examined (Kumar et al. 2017). For *P. fluorescens*, bacterial salt tolerance assay was conducted by inoculating *P. fluorescens* in nutrient broth supplemented with various concentrations of NaCl (0, 50, 100, 150, 200 and 250 mM of NaCl). The cultures were incubated at 25 ± 2 °C for 24 h. The absorbance was measured at 620 nm, and the cell density of *P. fluorescens* (cells/mL) at 24 h was compared (Egamberdieva et al. 2015). The tolerance level for isolates against different salt levels was determined based on the maximum salt concentration where growth is detected.

Biopriming seeds and their tolerance to salinity stress

The potting mix (coco peat, sand, burnt soil, burnt husk, rich humus, charcoal powder at pH 6) was first prepared and autoclaved twice (121 °C, 15 p.s.i). The potting mix was then kept in an oven (80 ± 2 °C) prior to transferring into polybags (200 g per polybag). Seeds were treated accordingly as either non-bioprimed (NB), bioprimed with *T. asperellum* (TAB), or bioprimed with *P. fluorescens* (PFB). To bioprime pak choy seeds, the seeds were first surface-sterilized with 1.5% sodium hypochlorite for 5 min, rinsed with sterile distilled water (Singh et al. 2016a), and air-dried under laminar airflow. Pak choy seeds were then imbibed in 1 mL of *T. asperellum* suspension (10^2 spores/mL of *T. asperellum* and 1.5% w:v sodium alginate) or in *P. fluorescens* suspension (10^7 cells/mL of *P. fluorescens* and 0.2% w:v xanthan gum) for 0.5 h and 1.0 h, respectively (Chin et al. 2021). The bioprimed seeds were air-dried on sterile filter paper for 3-4 h and sown into the potting mix.

The sown seeds were watered with distilled water (0 mM NaCl) for control treatment, or with salt solution (100 mM NaCl) to simulate saline soil conditions (SS). The electrical conductivity (EC) was read throughout the experimental period to ensure the non-saline soils (EC: 0.54 – 0.67 dS/m) and saline soils (EC: 2.49 – 3.05 dS/m) were at the appropriate recommended salinity range (Vargas et al. 2018). All treatments were placed in a plant house for one month and watered daily. Each pot consisted of ten seeds and one treatment had a total of

five pots. Pots were arranged in a randomized complete block design. The percentage of germination, root length, shoot length and fresh weight were recorded after one month of cultivation.

The protocol was repeated with chilli seeds, prepared as non-bioprimed chilli seeds (NB), chilli seeds bioprimed with *T. asperellum* (TAB: 10^2 spores/mL of *T. asperellum*, 1.5 % w:v sodium alginate, 1.0 h imbibition time), and chilli seeds bioprimed with *P. fluorescens* (PFB: 10^7 cells/mL of *P. fluorescens* and 0.2% w:v xanthan gum, 6.0 h imbibition time) (Chin et al. 2021; 2022a; 2022b). The seeds were treated similarly, with distilled water (control) or NaCl solution (to induce salinity stress). The germination percentage, root length, shoot length and fresh weight were recorded after one month of cultivation. All seedlings were then sampled for biochemical analysis to determine their physiological response to salinity stress.

Evaluation of proline content, total phenolic content and malondialdehyde lipid peroxidation level

Proline content in the plant tissues was assessed by first washing 0.5 g of seedlings with deionized water and homogenizing the tissues in 5 mL of 3% sulfosalicylic acid (Acros Organics, US) on ice bath. The extracts were filtered and centrifuged at 6000 rpm for 10 min (Eppendorf, Germany). The supernatant (2 mL) was collected and mixed with 2 mL of glacial acetic acid (Merck, Germany) and 2 mL of freshly prepared acid ninhydrin solution (R&M chemicals, UK). The mixture was boiled for 30 min and left to cool on ice bath prior to extraction with 4 mL of toluene (Merck, Germany). The absorbance was measured at 520 nm and the concentration of proline was calculated from the standard curve constructed using known concentrations of L-proline (Acros Organics, US) (Yu and Lu 2016; Zehra et al. 2017).

Total phenolic content in leaves was evaluated by placing 0.1 g of leaf tissue in 5 mL of 95% ethanol (J. Kollin, UK) and incubated for 48 h (4 ± 2 °C). The tissues were homogenized, and 1 mL of extract was reacted with 6.5 mL of mixture containing 1 mL of 95% ethanol, 5 mL of sterile distilled water and 0.5 mL of 50 % Folin-Ciocalteau reagent (Merck, Germany). After 5 min of incubation, 1 mL of 5 % sodium carbonate (Sigma-Aldrich, Germany) was added into the mixture and re-incubated for 1 h. The mixture was then read for absorbance at 725 nm. The final concentration of phenolics was calculated as mg of gallic acid equivalent (GAE) per 100 g of tissue fresh weight, derived from the standard curve of known concentrations of gallic acid (Friedemann Schmidt, Germany) (Rawat et al. 2011; Senguttuvan et al. 2014).

Malondialdehyde (MDA) lipid peroxidation levels in seedlings were examined by first homogenizing 0.25 g of leaf tissue in 5 mL of 0.1% trichloroacetic acid (TCA) (Merck, Germany). The extract (1 mL) was centrifuged (12000 rpm, 5 min) and mixed with 1 mL of 20% TCA containing 0.5% thiobarbituric acid (Cell Biolabs, USA). The mixture was heated (95 °C, 30 min) and left to cool on ice bath. The absorbance of the mixture was read at 532 nm and then at 600 nm, and the difference between the two absorbance readings was obtained through subtraction. The concentration of MDA was calculated according to Equation 1 (Rawat et al. 2012).

$$\text{Concentration of MDA} = (A532 - A600)/E \qquad \textit{(Eq. 1)}$$

where E, Extinction coefficient of MDA: 155 mM^{-1} cm^{-1}

Statistical analysis

All experiments were conducted in triplicates. Values are presented as means with standard error of means. Data was analysed with Analysis of Variance (ANOVA) using the Statistical Package for the Social Sciences (IBM SPSS) version 23.0. Means were compared with Tukey's Test ($HSD_{(0.05)}$).

Results and Discussion

Establishing salt-tolerance characteristics of isolates

T. asperellum and *P. fluorescens* demonstrated excellent salt-tolerant characteristics against high levels of salinity. *T. asperellum* tolerated high salinity stress, up to 250 mM of salt concentration, suggesting that salinity stress is not detrimental to *T. asperellum*. Nevertheless, slower colony growth was observed with increasing concentrations of salt. This was evident from the number of days required by *T. asperellum* to reach maximum growth diameter on the PDA plate, which was 1 day longer at higher salt concentrations (200 and 250 mM), compared to the lower concentrations (Supplementary Table A.1). Colony pigmentation was also influenced by increasing salt concentrations. *T. asperellum* cultured in PDA plates supplemented with 0 - 150 mM of salt (Fig. 1A - 1D) showed greenish colony pigmentation, while yellowish colony pigmentation was detected at higher salt concentrations (200 and 250 mM) (Fig. 1E and 1F). The yellowish-green colony pigmentation is deemed as a natural response to salt concentrations (up to 20 g/L, approx. 342 mM) in most *Trichoderma* spp. (Sánchez-Montesinos et al. 2019). The change in pigmentation of fungal mycelium was presumably due to the low laccase activity, as laccase is responsible for pigment synthesis. With laccase having sensitivity to high salt concentrations, pigmentation change is evident (Hölker et al. 2002). Despite the change in colony pigmentation, salt stress does not implicate fungal growth nor their biocontrol activi-

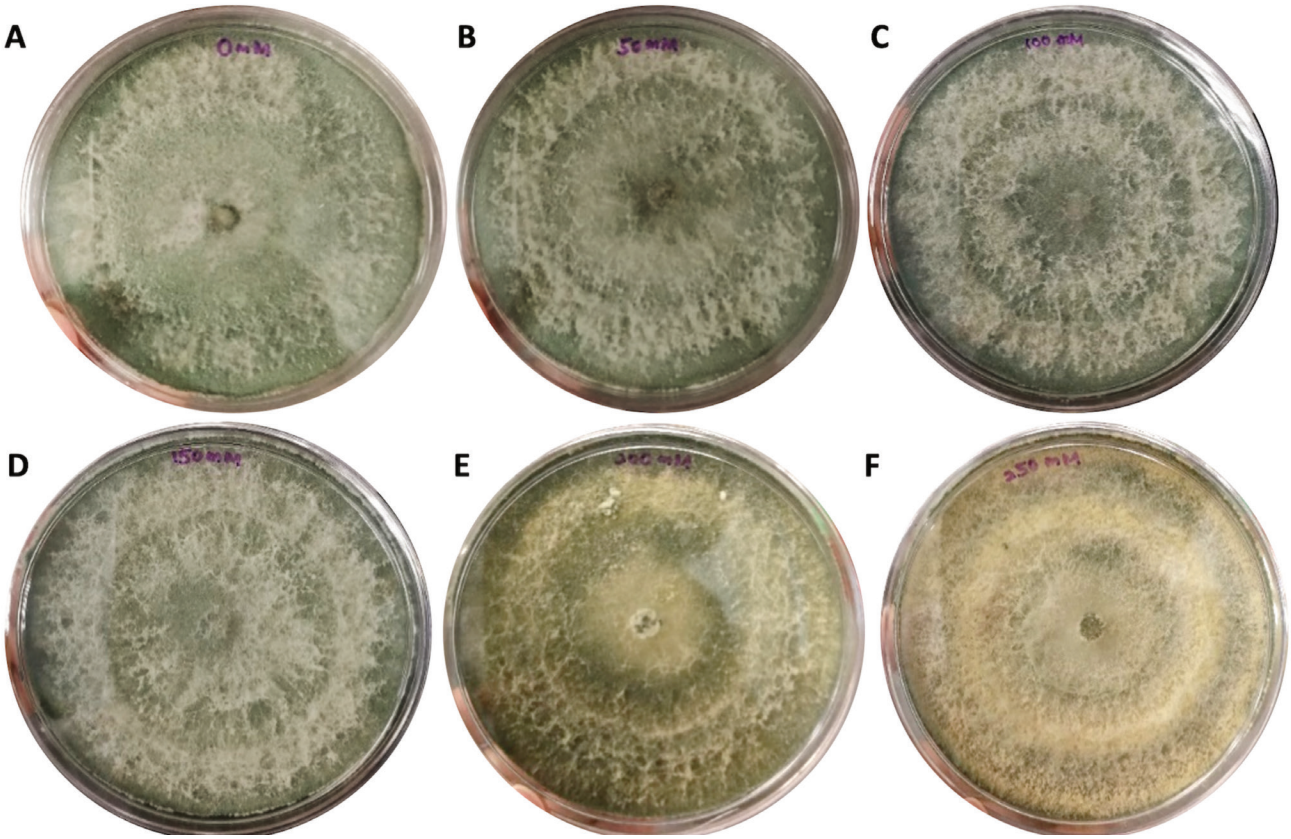

Figure 1. Macroscopic view of *Trichoderma asperellum* on Potato Dextrose Agar (PDA) supplemented with various salt concentrations (A: 0 mM, B: 50 mM, C: 100 mM, D: 150 mM, E: 200 mM, F: 250 mM) after one week of incubation.

ties (Poosapati et al. 2014). This most likely described the continuous growth of both *T. asperellum* and *P. fluorescens* in high salt concentrations.

P. fluorescens also showed adaptability to increasing salt concentrations (Fig. 2). *P. fluorescens* was able to grow in high salt concentrations, up to 250 mM with 2.294×10^8 cells/mL detected (Fig. 2). Growth of *P. fluorescens* in the presence of varying salt concentrations was significantly higher than in the absence of salt (control, 0 mM NaCl with only 1.943×10^8 cells/mL recorded) (Fig. 2). This indicated that *P. fluorescens* is capable of adapting, tolerating, and surviving in conditions with high salinity. The adaptability and tolerance of *P. fluorescens* is ascribed to physiological and biochemical adaptations. *Pseudomonas* spp. are known to accumulate solutes such as amino acids and sugars and produce extracellular polymeric substances (EPS) that help to capture salt ions (Pocard et al. 1994; Rajkumar et al. 2017). These mechanisms may have protected *P. fluorescens* from osmotic stress and promote water retention in bacteria, enabling survival in conditions with high salinity. Therefore, *T. asperellum* and *P. fluorescens* are suitable for the biopriming of vegetable seeds to promote seed tolerance against salinity stress.

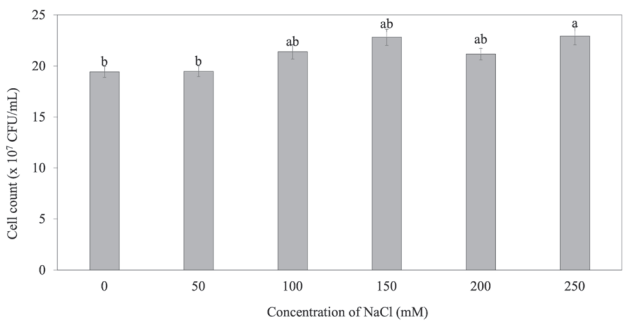

Figure 2. Bacterial cell count (× 10^7 CFU/mL) of *Pseudomonas fluorescens* in various salt concentrations (0 mM – 250 mM) after overnight incubation. Values are means of triplicates. Means with the same letters are not significantly different (One-way ANOVA, Tukey's Test, $p < 0.05$). Bars indicate standard error of means (± SEM).

Bioprimed seeds and their vegetative growth under salinity stress

Seedlings bioprimed with *T. asperellum* (TAB) and *P. fluorescens* (PFB) that were sown into saline soils, demonstrated different expressions to their vegetative growth and

Table 1. Growth characteristics of pak choy seedlings bioprimed according to various treatments and cultivated under salinity stress. Data are means ± standard error of mean from triplicates. Means with the same letters within a column are not significantly different (Tukey, $HSD_{(0.05)}$).

Treatment	Salinity treatment	Electrical conductivity (ds/m)	Germination percentage (%)	Mean root length (mm)	Mean shoot length (mm)	Mean fresh weight (g)
Non-bioprimed seeds (NB)	Non-saline soil	0.63 ± 0.06	60.00 ± 4.90^{a}	26.39 ± 3.70^{a}	36.15 ± 1.61^{a}	0.50 ± 0.05^{a}
	Saline soil	2.49 ± 0.10	32.00 ± 1.78^{b}	18.10 ± 1.45^{b}	31.43 ± 2.32^{a}	0.16 ± 0.01^{c}
Seeds bioprimed with T. asperellum (TAB)	Non-saline soil	0.67 ± 0.05	54.00 ± 6.69^{a}	24.94 ± 1.41^{a}	39.31 ± 2.48^{a}	0.52 ± 0.04^{a}
	Saline soil	3.05 ± 0.54	38.00 ± 7.16^{b}	19.50 ± 2.97^{b}	29.69 ± 3.05^{a}	0.59 ± 0.18^{a}
Seeds bioprimed with P. fluorescens (PFB)	Non-saline soil	0.54 ± 0.04	60.00 ± 9.80^{a}	24.56 ± 2.48^{a}	36.71 ± 2.00^{a}	0.42 ± 0.13^{a}
	Saline soil	2.72 ± 0.17	28.00 ± 6.57^{b}	18.28 ± 1.32^{b}	34.02 ± 1.35^{a}	0.23 ± 0.02^{a}

germination percentage. Bioprimed pak choy seedlings (TAB, PFB) cultivated in saline soils, benefited the most from the association with salt-tolerant isolates. Enhanced fresh weights were observed, while the shoot lengths, root lengths and germination percentages were comparable to non-bioprimed seedlings (NB). Fresh weight of TAB seedlings in saline soils were significantly higher (0.59 ± 0.18 g) compared to non-bioprimed (NB) and PFB seedlings in similar saline soils (0.16 ± 0.01g and 0.23 ± 0.02 g, respectively) (Table 1, Supplementary Fig. A.1). TAB seedlings also had slightly higher germination percentages (38.00 ± 7.16%) although this was not significantly different from NB (32.00 ± 1.78%) and PFB (28.00 ± 6.57%) seedlings (Table 1). Similarly, root lengths for TAB seedlings were relatively longer (19.50 ± 2.97 mm) but were not significantly different from PFB (18.28 ± 1.32 mm) and NB (18.10 ± 1.45 mm) seedlings. For shoot lengths, TAB seedlings recorded lower shoot lengths (29.69 ± 3.0 mm) compared to PFB (34.02 ± 1.35 mm) and NB (31.43 ± 2.32 mm) seedlings, although shoot lengths of seedlings in all three treatments were not significantly different (Table 1).

In general, *T. asperellum* (TAB) and *P. fluorescens* (PFB) were concluded to be compatible with pak choy as there were no implications to growth and germination of the seedlings that were detected. This was summarized from observations in non-saline soil conditions where the germination percentages, root lengths, shoot lengths, and fresh weight of seedlings were not significantly different among all three treatments. TAB seedlings recorded 54.00 ± 6.69% germination rate, comparable to the 60% germination rate for both PFB and NB seedlings (Table 1). TAB and PFB seedlings also have root lengths (24.56-24.94 mm), shoot lengths (36.71-39.31 mm), and fresh weights (0.42-0.52 g fr. wt.) that were similar to NB seedlings (26.39 ± 3.70 mm, 36.15 ± 1.61 mm, and 0.50 ± 0.05 g fr. wt) (Table 1).

For chilli seedlings, biopriming with *T. asperellum* (TAB) and *P. fluorescens* (PFB) significantly enhanced shoot lengths, while other growth parameters and germination rates were relatively comparable. The TAB and PFB seedlings showed higher shoot lengths of 32.79 ± 2.92 mm and 32.39 ± 1.92 mm, respectively, compared to 26.86 ± 3.68 mm for NB seedlings (Table 2, Supplementary Figure A.2). The bioprimed seedlings however, had slightly lower root lengths and fresh weight of seedlings compared to non-bioprimed (NB) seedlings, although these values were not significantly different. The root length of TAB and PFB seedlings were not significantly different from NB seedlings, with 34.84 ± 1.56 mm, 31.97 ± 1.63 mm, 36.05 ± 4.71 mm, respectively. Similarly, the fresh weight of seedlings was not significantly different with 0.09 - 0.11 g fr. wt. recorded for seedlings in all three treatments. The only aspect that was contrasting was the germination percentage for TAB (62.00 ± 11.58%) seedlings, which

Table 2. Growth characteristics of chilli seedlings bioprimed according to various treatments and cultivated under salinity stress. Data are means ± standard error of mean from triplicates. Means with the same letters within a column are not significantly different (Tukey, $HSD_{(0.05)}$).

Treatment	Salinity treatment	Electrical conductivity (ds/m)	Germination percentage (%)	Mean root length (mm)	Mean shoot length (mm)	Mean fresh weight (g)
Non-bioprimed seeds (NB)	Non-saline soil	0.63 ± 0.06	86.00 ± 6.00^{a}	47.64 ± 2.03^{a}	28.56 ± 1.24^{b}	0.15 ± 0.02^{ab}
	Saline soil	2.49 ± 0.10	82.00 ± 9.17^{a}	36.05 ± 4.71^{ab}	26.86 ± 3.68^{b}	0.11 ± 0.01^{ab}
Seeds bioprimed with T. asperellum (TAB)	Non-saline soil	0.67 ± 0.05	78.00 ± 5.83^{ab}	44.43 ± 4.58^{ab}	34.75 ± 1.38^{a}	0.14 ± 0.01^{ab}
	Saline soil	3.05 ± 0.54	62.00 ± 11.58^{b}	34.84 ± 1.56^{ab}	32.79 ± 2.92^{a}	0.10 ± 0.01^{b}
Seeds bioprimed with P. fluorescens (PFB)	Non-saline soil	0.54 ± 0.04	86.00 ± 2.45^{a}	48.44 ± 2.41^{a}	29.61 ± 2.43^{b}	0.18 ± 0.02^{a}
	Saline soil	2.72 ± 0.17	78.00 ± 9.70^{ab}	31.97 ± 1.63^{b}	32.39 ± 1.92^{a}	0.09 ± 0.02^{b}

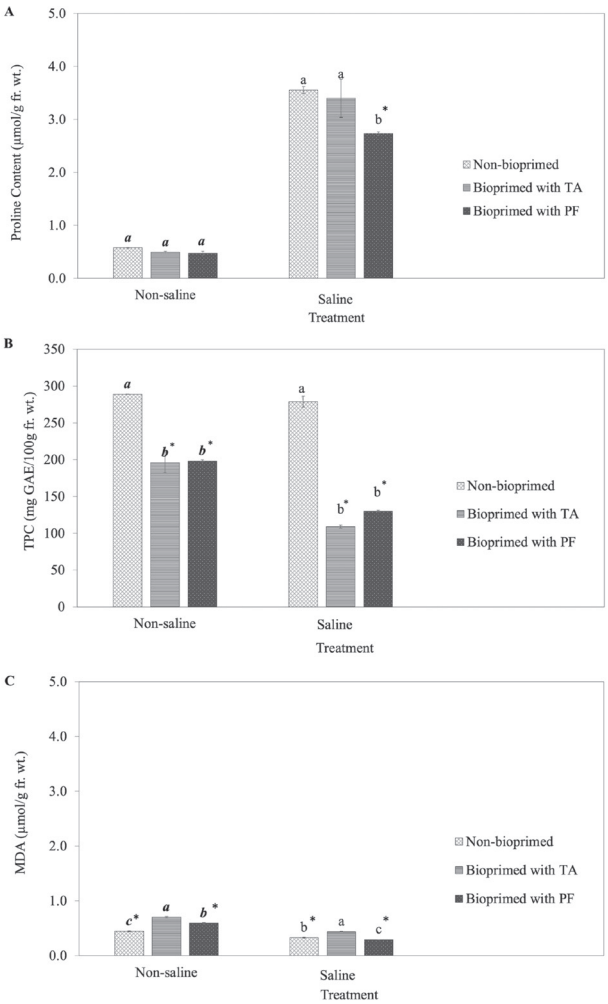

Figure 3. (A) Proline content, (B) total phenolic content (TPC), (C) malondialdehyde (MDA) level in pak choy seedlings, grew in non-saline soils and saline soils. NB: Non-bioprimed seeds, TAB: Seeds bioprimed with *Trichoderma asperellum*, PFB: Seeds bioprimed with *Pseudomonas fluorescens*. Data are means ± standard error of mean from triplicates. Means with the same letters and caption are not significantly different (One-way ANOVA, Tukey's Test, $p < 0.05$). * Indicates significant difference to non-bioprimed seed with respective stress condition (unpaired T-test, $p < 0.05$).

was significantly lower than NB (82.00 ± 9.17%) and PFB (78.00 ± 9.70%) seedlings (Table 2).

T. asperellum (TAB) and *P. fluorescens* (PFB) are assumed to be able to render beneficial effect to chilli seedlings, with *P. fluorescens* having a more likely positive association. In non-saline soils, the growth of PFB seedlings were better with relatively higher root lengths (48.44 ± 2.41 mm), fresh weights (0.18 ± 0.02 g fr. wt.) and germination rates (86.00 ± 2.45%). This is in comparison to NB seedlings with 47.64 ± 2.03 mm, 0.15 ± 0.02 g fr. wt. and 86.00 ± 6.00%, respectively. On the other hand, biopriming with *T. asperellum* led to higher shoot length (34.75 ± 1.38 mm) (Table 2).

The germination and growth of bioprimed seedlings under salinity stress, as well as in control conditions (non-saline soils), revealed several key findings. Firstly, the salinity-tolerant isolates (*T. asperellum* and *P. fluorescens*) were established to be compatible with the vegetable seeds. Both pak choy and chilli seedlings germinated and grew without severe implications from the close association with *T. asperellum* and *P. fluorescens*. Improved vegetative growth has been reported in other studies involving species of *Trichoderma* and *Pseudomonas*. Tolerance to salinity stress was observed in wheat seedlings bioprimed with *T. harzanium*, with enhanced germination percentage, root and shoot lengths of the wheat seedlings (Rawat et al. 2011). Similarly, the germination rate of *Arabidopsis* seeds bioprimed with *Pseudomonas* spp. was enhanced (more than 50%) under salinity stress (150 mM NaCl) (Chu et al. 2019). The plant-growth promotion is correlated with the production of indole acetic acid (IAA), 1-aminocyclopropane-1-carboxylate deaminase and other phytohormones regulated by *Trichoderma* and *Pseudomonas* spp., which enhanced nutrient and water uptake by plants (Dhananjaya Pratap et al. 2016).

The influence of *T. asperellum* and *P. fluorescens* were more encouraging for pak choy than for chilli seedlings. For chilli seedlings, biopriming with *T. asperellum* and *P. fluorescens* appeared to be less effective than non-bioprimed seedlings. This clearly indicated that while *T. asperellum* and *P. fluorescens* are generalists and broad-spectrum beneficial isolates, they may still elicit different responses in different plant-microbe associations. As such, it is pertinent that every seed biopriming exercise is performed via careful selection, testing and optimization using different isolates. This study also revealed that among the various growth factors, the root length of seedlings is severely impacted by salinity stress (NB, TAB, PFB in saline soil) compared to non-saline soils, with 18.10-19.50 mm compared to 24.56-26.39 mm for pak choy, and 31.97-36.05 mm compared to 44.43 to 48.44 mm for chilli seedlings, respectively (Table 1, 2). The impact of salinity on root elongation observed in this study agrees with Hakim et al. (2010) where root and shoot length of *Oryza sativa* L. decreased under the influence of salinity stress (20 dS/m). A shorter root system may diminish nutrient uptake, leading to nutritional imbalance and substantial influence on plant growth (Singh et al. 2019).

However, unlike Hakim et al. (2010), our study demonstrated that the shoot length of chilli seedlings was improved with biopriming using *T. asperellum* (TAB). Shoot length was the highest when bioprimed with *T. asperellum* (TAB) for seedlings cultivated in both the absence (34.75 ± 1.38 mm) and presence (32.79 ± 2.92 mm) of salinity stress (Table 2). Enhanced shoot growth is likely attributed to

the possible production of auxin-like compounds, which are known to be produced by species of *Trichoderma*. Contreras-Cornejo et al. (2009) further evidenced this with reports of *T. virens* and *T. atroviride* promoting shoot growth (50%) due to the auxin-like compounds (i.e., IAA, indole-3-acetaldehyde, indole-3-ethanol) produced.

Bioprimed seeds and their physiological response under salinity stress

For pak choy, the stress-related biochemical markers revealed that biopriming with *P. fluorescens* rendered protection to pak choy seedlings (PFB) under salinity stress. This was marked by the lower levels of proline (2.73 µmol/g fr. wt.), total phenolic content (TPC) (130.10 mg GAE/100g ft. wt.) and malondialdehyde (MDA) (0.29 µmol/g fr. wt.), which were significantly lower than non-bioprimed seedlings (NB) (Fig. 3). This observation suggested that PFB seedlings experienced less stressful conditions when exposed to salinity stress. *T. asperellum* was however, less effective in promoting tolerance of pak choy (TAB) against salinity stress, as indicated by the higher levels of proline (3.40 µmol/g fr. wt.) and MDA (0.44 µmol/g fr. wt.), compared to the non-bioprimed seedlings (NB, control) (Fig. 3). Nevertheless, the vegetative growth of *T. asperellum*-bioprimed seedlings (TAB) was not severely impacted by the increased biochemical markers, postulating that the plant-growth regulators from *T. asperellum* may have countered the effects by the plant stress. The plant-growth-promoting properties of *Trichoderma* spp. has been widely studied in other crops such as wheat, resulting in improved plant growth and water use efficiency under salinity stress (200 mM) (Oljira et al. 2020). Oljira et al. (2020) also found that wheat bioprimed with *Trichoderma yunnanense* and *Bacillus licheniformis* had a lower level of proline under stressful conditions, but the effects vary according to the seed cultivars and microbial variants. It has also been suggested that *Trichoderma* spp. stimulate plant growth under salinity stress by secreting indole-3-acetic acid (IAA) to counteract the increasing ethylene concentration in plants, thereby improving net photosynthesis and promoting water uptake at the root system (Nadeem et al. 2014; Oljira et al. 2020).

In conditions without salinity stress, pak choy seeds bioprimed with *T. asperellum* (TAB) and *P. fluorescens* (PFB) showed lower levels of proline (0.47 – 0.58 µmol/g fr. wt.) and TPC (195.88 - 198.24 mg GAE/100g ft. wt.), but a significantly higher level of MDA (TAB and PFB, 0.59 - 0.70 µmol/g fr. wt.) when compared to non-bioprimed seedlings (NB) (Fig. 3). Plants experiencing stress generally produce more inorganic ions and osmolytes such as proline to regulate the osmotic potential in plant cells (Verslues and Sharma 2010; Li et al. 2019). The production of phenolic compounds also increases in parallel to the induced resistance mechanism, to control oxidative stress when plants are exposed to stresses and microbial inoculation (Zheng and Shetty 2000; Boughalleb et al. 2020; Wallis and Galarneau 2020). For malondialdehyde, the indicator of cell lipid peroxidation level increases when higher levels of cellular oxidative stress occur due to stress (Boughalleb et al. 2020). Therefore, increased oxidative stress (high MDA level) is an indicator of stress response towards environmental factors. Among the three different biochemical markers, the enhanced levels of proline in seedlings under salinity stress is the most evident. As proline is expressed under salinity stress, it is concluded that pak choy seedlings produced more proline to act as an osmolyte to buffer the movement of Na ions.

For chilli seedlings, *T. asperellum* and *P. fluorescens* was less effective in rendering salinity tolerance to chilli seedlings (TAB, PFB), although levels of proline (0.61 - 1.04 µmol/g fr. wt.), TPC (80.00 - 80.29 mg GAE/100g ft. wt.) and malondialdehyde (0.48 - 0.61 µmol/g fr. wt.) were low (Fig. 4). These biochemical markers were significantly lower than non-bioprimed seedlings (NB, proline: 1.87 µmol/g fr. wt.; TPC: 105.59 mg GAE/100g ft. wt., MDA: 0.61 µmol/g fr. wt.). However, these observations did not correspond to their vegetative growth, suggesting that growth may have been compromised as a stress response to salinity stress. In the absence of salinity stress, *T. asperellum* caused a slight increase of proline (0.91 µmol/g fr. wt.), TPC (118.14 mg GAE/100g ft. wt.) and MDA (0.68 µmol/g fr. wt.) levels in bioprimed seedlings (TAB) when compared to non-bioprimed seedlings (NB) (Fig. 4).

Therefore, based on both vegetative growth and physiological responses, our study suggested that pak choy benefited from biopriming using *P. fluorescens*, as the seedlings showed characteristics of being buffered from direct exposure to salinity (Egamberdieva et al. 2015). This may be attributed to *Pseudomonas* spp. producing exopolysaccharides (EPS) which may bind sodium cations on the cell surface. By lowering the availability of sodium in soil, the host plant has lesser exposure to high concentrations of salt (Singh et al. 2019). Additionally, accumulation of proline and antioxidant enzymes in black gram and maize seeds inoculated with salt-tolerant *Pseudomonas* spp. under salinity stress have also been detected as a response to the high levels of salts in soil (Raja et al. 2017; Singh et al. 2019). The benefits of biopriming with *Pseudomonas* spp. to promote tolerance to salinity have also been observed in other crop types such as *Brassica juncea* (Phour and Sindhu 2020), eggplant (Fu et al. 2010), and cotton (Egamberdieva et al. 2015).

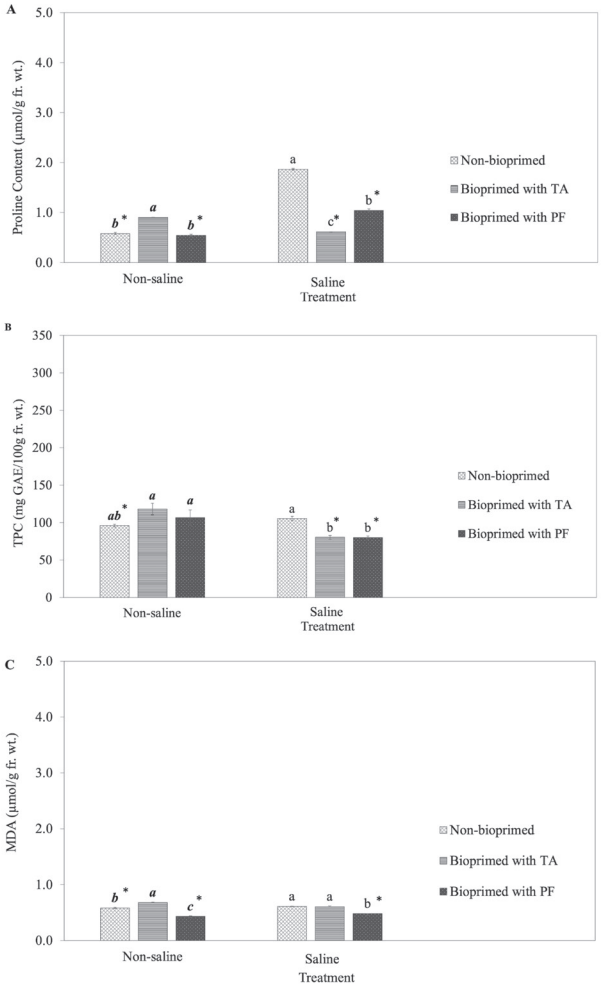

Figure 4. (A) Proline content, (B) total phenolic content (TPC), (C) malondialdehyde (MDA) level in chilli seedlings, grew in non-saline soils and saline soils. NB: Non-bioprimed seeds, TAB: Seeds bioprimed with *Trichoderma asperellum*, PFB: Seeds bioprimed with *Pseudomonas fluorescens*. Data are means ± standard error of mean from triplicates. Means with the same letters and caption are not significantly different (One-way ANOVA, Tukey's Test, $p < 0.05$). * Indicates significant difference to non-bioprimed seed with respective stress condition (unpaired T-test, $p < 0.05$).

Conclusions

This study revealed that *T. asperellum* and *P. fluorescens* have salt-tolerant characteristics as established from the *in vitro* assay. When bioprimed to seeds, they rendered some form of protection to pak choy and chilli seedlings against salinity stress. Among the bioprimed seed treatments, *P. fluorescens* showed potential in rendering better tolerance for pak choy seeds towards salinity stress compared to *T. asperellum*, evident by lower levels of stress-related biochemical markers. *P. fluorescens* may have protected the seedlings from salt toxicity, ensuring subsequent growth of seedlings. Our findings provided a glimpse into the potential application of salt-tolerant microorganisms for seeds. However, further studies are required to ascertain the role of salt-tolerant isolates in microbe-mediated salinity tolerance in host plants.

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