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Altered physiological response in drought stressed rice plants upon root colonization with the beneficial endophytic fungus *Piriformospora indica* under field conditions

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Abstract

We studied the physiological and biochemical responses of rice plants colonized by the root endophytic fungus *Piriformospora indica* under varying moisture stress levels that consisted of ideal (non-stress), mild, moderate, severe, very severe, and extremely severe stress imposed by altering depth of irrigation and frequency, in a summer field crop. Colonization by *P. indica* exhibited distinctive drought defensive effects characterized by the enhanced production of proline, which contributed to improved plant resilience to drought stress, alleviating the harmful oxidative stress. In colonized plants that were under extremely severe stress, proline levels in leaf tissues rose by 18% during panicle initiation (PI) and by 21% during the flowering stage, compared to the uninoculated plants. *P. indica* colonization also enhanced the relative leaf water content and cell membrane stability in plants. Under extremely severe stress, colonized plants displayed improved cell membrane stability (57% and 48%) at PI and flowering, representing 29% and 8% improvement, respectively, over the non-colonized plants under stress. Endophyte colonized plants demonstrated increased resistance to drought stress with enhanced chlorophyll stability when compared to stressed plants that were not colonized. Fungal colonization also enhanced the growth and resilience of rice plants under drought, resulting in a remarkable 37% increase in grain yield compared to non-colonized plants.

Keywords

biochemical response; cell membrane stability; chlorophyll stability; drought stress; proline; relative leaf water content; root endophyte

Introduction

Plant-microbe associations that improve the host's tolerance to abiotic and biotic stress have become a major focus in eco-friendly agriculture. *Piriformospora indica* is a beneficial fungus isolated from the Thar desert in Rajasthan, India (1) belonging to the Sebaciales that (2) forms a mutualistic association with host plants by colonizing the intercellular or intracellular spaces of root tissues. *P. indica* colonized plants exhibited increased biomass accumulation and nutrient uptake (3), improved tolerance to drought (4) and disease resistance (5, 6). *P. indica* positively influenced water use efficiency, photosynthetic rate, stomatal conductance, and carboxylation efficiency in rice plants (7).

Beneficial interaction with *P. indica* activated glutathione-ascorbate redox pathway (8), while it enhanced antioxidant enzyme activity and metabolic rates in barley leaves under salt stress (9). Its interaction also resulted in elevated catalase (CAT) and superoxide dismutase (SOD) activity, strengthening the plant defense system and reducing vulnerability to biotic stress in maize (10). *P. indica* colonization resulted in elevated chlorophyll content in black pepper (11). *P. indica* colonization in maize increased stomatal conductance, CO₂ accumulation, and chlorophyll content under salt stress, mitigating negative effects compared to uncolonized plants (12). *P. indica* colonized rice exhibited an increase in levels of chl-*a*, *b*, and carotene pigments compared to uninoculated plants under salt stress (13).

Higher levels of proline and relative water content are reported in the endophyte inoculated rice plants under salt stress compared to noncolonized plants (14). The researchers observed increased chlorophyll content in leaves and sugar content in grains in *P. indica* colonized rice (15). Additionally, increased total soluble sugar and soluble protein with the endophyte over non-colonized sweet potato plants were also observed (16). In an effort to decipher the basis of *P. indica* mediated moisture stress tolerance in rice, we assessed the physiological and biochemical parameters acquired from the leaves of colonized and non-colonized rice plants under varied levels of moisture stress.

Materials and Methods

The seedlings of the rice variety MO 21 were co-cultured with the endophytic fungus in the nursery before transplanting, following the previously described method (17). Surface sterilization of the rice seed was performed by soaking in a solution containing 0.1% HgCl₂ for 1 min, followed by rinsing in sterile water twice. The seeds were further soaked in sterile distilled water for 12 h and co-cultured as described elsewhere (18). Evaluation of root colonization involved the selection of 10 randomly chosen root fragments from the inoculated plants at 5 days after sowing, cut into approximately 1 cm in length. The presence of the fungal chlamydo spores after staining was ascertained by examining these root fragments under a light microscope (11). Surface sanitized seeds soaked in distilled water and sown in rooting medium devoid of the fungus served as the control group.

A summer season rice crop was raised in lowland situated at 8° 43' N latitude and 76° 98' E longitude, with an altitude of 20.0 m above mean sea level with low available nitrogen, high available phosphorus, and medium available potassium. The experiment involved transplanting 2-weeks old inoculated (P₁) and non-inoculated (P₀) rice plants into 20 m² plots at a spacing of 15 cm × 10 cm. The plants were subjected to varying levels of moisture stress, with two depths of irrigation (1.5 cm and 3 cm) and three irrigation frequencies based on the cumulative pan evaporation values (CPE) (30 mm, 35 mm, and 40 mm). The experimental design that followed was a

factorial randomized block design with three replications. Different levels of moisture stress were imposed on plants, categorized as ideal/non-stress/normal (MS0: 30 mm CPE, depth 3 cm), mild (MS1: 35 mm CPE, depth 3 cm), moderate (MS2: 40 mm CPE, depth 3 cm), severe (MS3: 30 mm CPE, depth 1.5 cm), very severe (MS4: 35 mm CPE, depth 1.5 cm), and extremely severe (MS5: 40 mm CPE, depth 1.5 cm). Evaporation data on a daily basis was utilized to determine the irrigation frequency, and a water meter (Kranti, India) was used to gauge the amount of irrigation water to be administered to each plot according to the designated treatments. A fully emerged third leaf from the top was taken from the field from 3 plants within each plot at panicle initiation (PI) and flowering stage for the estimation of proline (19), relative leaf water content (RLWC) (20) and cell membrane stability index (CMSI) (21). Chlorophyll stability index (CSI) was estimated using the previously given formula (22) from the fresh leaf samples.

Results and Discussion

The physiological and biochemical basis of tolerance to moisture stress could be understood in terms of proline content, RLWC, CMSI and CSI (23-25). Enhancement of drought tolerance through differential biochemical response upon *Piriformospora indica* colonization was observed with respect to these physiological parameters and was reflected on the yield. Plants experiencing optimal conditions or lacking any moisture-related stress recorded the highest RLWC, CMS and CSI (Table 1-3).

P. indica colonization displayed an osmoprotectant signature of increase in proline content for plant tolerance to drought stress. Plants inoculated with the endophyte under extremely severe stress had the highest proline content over non-colonized ones experiencing the same level of stress. It was increased by 18.09 and 21.37% at PI and flowering stages in inoculated plants at extremely severe stress over non-colonized plants (Fig. 1). An inverse relationship between water content in soil and proline accumulation in leaves of rice was observed by Dien *et al.* (26). Proline is an osmoprotectant having major role in safeguarding many enzymes and is an important source of cellular carbon and nitrogen. Its production favorably enhances drought stress mitigation by inducing osmotic adjustments by plant cells, and a quantitative measurement of the same in colonized rice plants could prove the same. Enhancement of proline content in colonized plants might be due to the upregulation of mi RNA with biotization (27). It was evident that co-cultivation of *P. indica* enhanced reactive oxygen species (ROS) scavenging/signaling which reduced ROS thereby reducing the oxidative damage to cells directed to drought tolerance (28).

Relative leaf water content is a prime indicator of water status in plants, indicating their adaptability to stress conditions. This parameter is used for the determination of drought tolerance, indicating the level of protoplast hydration for extended periods (29). Root endophyte inoculated plants irrigated under no stress

Table 1. Effects of *P. indica* colonization, irrigation interval and depth of irrigation on physiological and biochemical parameters associated with stress mitigation in rice.

Treatments	Proline ($\mu\text{mole g}^{-1}$)		Chlorophyll Stability Index (%)		Relative Leaf Water Content (%)		Cell Membrane Stability Index (%)	
	PI	Flowering	PI	Flowering	PI	Flowering	PI	Flowering
P₁MS₀	55.67 ± 2.08 ^h	46.33 ± 2.52 ^{hi}	95.80 ± 0.36 ^a	88.63 ± 0.85 ^a	87.93 ± 0.35 ^a	85.13 ± 0.45 ^a	95.00 ± 1.61	85.00 ± 1.61 ^a
P₁MS₁	66.67 ± 2.52 ^{fg}	49.00 ± 3.61 ^{gh}	84.93 ± 1.80 ^b	81.23 ± 0.45 ^c	80.87 ± 0.47 ^b	77.67 ± 0.55 ^c	88.60 ± 2.15	81.27 ± 0.51 ^b
P₁MS₂	69.67 ± 1.53 ^f	59.33 ± 1.53 ^f	80.23 ± 0.57 ^c	76.93 ± 0.75 ^d	78.27 ± 0.57 ^c	75.10 ± 0.75 ^d	83.20 ± 1.77	76.53 ± 0.40 ^d
P₁MS₃	85.67 ± 1.53 ^d	74.67 ± 2.08 ^d	76.20 ± 0.30 ^e	65.87 ± 0.45 ^g	76.10 ± 0.30 ^d	72.17 ± 0.55 ^e	68.17 ± 1.76	61.17 ± 0.55 ^g
P₁MS₄	115.33 ± 2.52 ^a	106.00 ^a ± 2.00	66.20 ± 0.36 ^h	49.90 ± 0.70 ⁱ	68.70 ± 0.72 ^f	65.10 ± 0.80 ^h	58.86 ± 0.88	47.50 ± 0.75 ^j
P₁MS₅	105.67 ± 1.53 ^b	95.67 ± 1.53 ^b	71.83 ± 0.40 ^g	57.50 ± 1.06 ⁱ	73.13 ± 0.55 ^e	68.85 ± 0.58 ^f	63.17 ± 1.56	52.50 ± 0.95 ⁱ
P₀MS₀	41.67 ± 1.53 ⁱ	42.67 ± 1.53 ^j	84.17 ± 0.85 ^b	82.77 ± 1.11 ^b	80.80 ± 0.40 ^b	80.86 ± 0.67 ^b	88.10 ± 1.28	78.77 ± 0.40 ^c
P₀MS₁	58.33 ± 2.52 ^h	45.00 ± 1.00 ^{ij}	78.53 ± 0.40 ^d	72.00 ± 1.04 ^e	77.77 ± 0.51 ^c	75.97 ± 0.57 ^d	82.20 ± 2.82	71.87 ± 0.70 ^e
P₀MS₂	64.00 ± 2.00 ^g	51.00 ± 1.00 ^g	73.73 ± 0.35 ^f	68.80 ± 0.46 ^f	76.47 ± 0.40 ^d	73.13 ± 0.74 ^e	78.23 ± 1.99	67.90 ± 0.46 ^f
P₀MS₃	76.33 ± 0.58 ^e	64.00 ± 2.00 ^e	67.13 ± 0.35 ^h	59.33 ± 0.25 ^h	73.77 ± 0.47 ^e	67.70 ± 0.56 ^g	61.47 ± 2.06	54.80 ± 1.31 ^h
P₀MS₄	97.67 ± 1.53 ^c	87.33 ± 1.53 ^c	54.13 ± 0.80 ⁱ	43.90 ± 0.87 ^k	63.23 ± 1.15 ^g	60.13 ± 0.45 ⁱ	50.36 ± 0.80	43.90 ± 2.43 ^k
P₀MS₅	87.67 ± 1.53 ^d	72.67 ± 1.53 ^d	61.03 ± 1.37 ⁱ	51.10 ± 0.78 ^j	69.63 ± 0.45 ^f	64.20 ± 0.56 ^h	54.57 ± 1.85	45.23 ± 1.19 ^k
SEm (\pm)	1.08	1.12	0.46	0.45	0.33	0.35	1.03	0.64
CD (0.05)	3.14	3.28	1.35	1.31	0.96	1.03	-	1.86

P₁MS₀: colonised plants under no stress, **P₀MS₀**: control plants under no stress, **P₁MS₁**: colonised plants with mild stress, **P₀MS₁**: control plants under mild stress, **P₁MS₂**: colonised plants under moderate stress, **P₀MS₂**: control plants under moderate stress, **P₁MS₃**: colonised plants under severe stress, **P₀MS₃**: control plants under severe stress, **P₁MS₄**: colonised plants under very severe stress, **P₀MS₄**: control plants under very severe stress, **P₁MS₅**: colonised plants under extremely severe stress, **P₀MS₅**: control plants under extremely severe stress

Each data represents mean (\pm SD) of 3 replications (n=15). Figures in a column followed by same letter do not differ significantly

Table 2. Effect of *P. indica* colonization, irrigation interval and depth of irrigation on physiological and biochemical parameters associated with stress mitigation.

Treatments	Proline ($\mu\text{mole g}^{-1}$)		Chlorophyll Stability Index (%)		Relative Leaf Water Content (%)		Cell Membrane Stability Index (%)	
	PI	Flowering	PI	Flowering	PI	Flowering	PI	Flowering
P ₁ (<i>P. indica</i> colonized rice)	83.11	71.83	77.50	74.00	75.82	67.32	79.20	70.01
P ₀ (non-colonized rice)	70.94	60.44	73.61	70.33	68.07	60.41	69.78	62.98
SEm (\pm)	0.46	0.40	0.13	0.15	0.44	0.26	0.19	0.18
CD (0.05)	1.343	1.185	0.384	0.441	1.296	0.755	0.549	0.535
Irrigation interval (CPE)								
30 mm	64.83	56.91	79.65	76.46	78.18	69.93	80.82	74.15
35 mm	79.58	65.58	75.35	71.67	72.13	62.71	74.08	65.45
40 mm	86.66	75.91	71.66	68.36	65.54	58.95	68.57	59.88
SEm (\pm)	0.56	0.49	0.16	0.18	0.54	0.31	0.23	0.22
CD (0.05)	1.644	1.451	0.470	0.541	1.588	0.924	0.672	0.655
Depth of irrigation								
1.5 cm	94.72	83.38	70.76	66.35	58.01	50.85	66.08	54.60
3 cm	59.33	48.88	80.35	77.97	85.88	76.88	82.90	78.39
SEm (\pm)	0.46	0.40	0.13	0.15	0.44	0.26	0.19	0.18
CD (0.05)	1.343	1.185	0.384	0.441	1.296	0.755	0.549	0.535

Table 3. Interaction effect of *P. indica* colonization, irrigation interval and depth of irrigation on physiological and biochemical parameters associated with stress mitigation.

Treatments	Proline ($\mu\text{mole g}^{-1}$)		Chlorophyll Stability Index (%)		Relative Leaf Water Content (%)		Cell Membrane Stability Index (%)	
	PI	Flowering	PI	Flowering	PI	Flowering	PI	Flowering
P × CPE interaction								
Colonized, 30 mm	70.66	60.50	82.01	78.65	81.58	73.08	86.00	77.25
Colonized, 35 mm	86.16	72.33	77.00	73.25	75.88	66.88	78.38	69.36
Colonized, 40 mm	92.50	82.66	73.48	70.10	70.01	62.01	73.21	63.41
Non-colonized, 30 mm	59.00	53.33	77.28	74.27	74.78	66.78	75.65	71.05
Non-colonized, 35 mm	73.00	58.83	73.70	70.08	68.38	58.55	69.78	61.55
Non-colonized, 40 mm	80.83	69.16	69.85	66.63	61.06	55.90	63.93	56.35
SEm (\pm)	0.79	0.70	0.23	0.26	0.76	0.45	0.32	0.32
CD (0.05)	2.325	2.052	0.667	0.765	2.245	1.307	0.951	0.926
P × Depth interaction								
Colonized, 1.5 cm	102.22	92.11	72.64	68.70	62.72	53.72	71.41	57.75
Colonized, 3 cm	64.00	51.55	82.35	79.30	88.93	80.93	86.98	82.26
Non-colonized, 1.5 cm	87.22	74.66	68.87	64.01	53.31	47.97	60.76	51.44
Non-colonized, 3 cm	54.66	46.22	78.34	76.65	82.84	72.84	78.81	74.52
SEm (\pm)	0.65	0.57	0.18	0.21	0.62	0.36	0.26	0.26
CD (0.05)	1.899	1.676	0.545	0.624	1.833	1.067	0.776	0.756
CPE × Depth interaction								
30 mm, 1.5 cm	81.00	69.33	74.93	69.93	64.81	57.98	71.66	62.60
35 mm, 1.5 cm	96.66	84.16	71.38	66.52	58.86	48.86	66.43	54.30
40 mm, 1.5 cm	106.50	96.66	65.96	62.61	50.36	45.70	60.16	46.90
30 mm, 3 cm	48.66	44.50	84.36	82.99	91.55	81.88	89.98	85.70
35 mm, 3 cm	62.50	47.00	79.31	76.82	85.40	76.56	81.73	76.61
40 mm, 3 cm	66.83	55.16	77.36	74.11	80.71	72.21	76.98	72.86
SEm (\pm)	0.79	0.70	0.23	0.26	0.76	0.45	0.32	0.32
CD (0.05)	2.325	2.052	0.667	0.765	2.245	1.307	0.951	0.926

conditions recorded the highest RLWC of 87.93 and 85.13% at PI and flowering, respectively (Fig. 1). Uninoculated rice plants under severe stress produced the lowest RLWC (63.23 and 60.13% at PI and flowering) due to lower water status in the leaves. Jahan *et al.* (30) attributed lower RLWC to the increase in osmotic potential of the cells. *P. indica* colonized plants subjected to extremely severe stress maintained higher RLWC, showing an improvement of 8.6 and 8.26% over stressed control plants at PI and flowering stage. The commencement of panicle formation has a determining role in determining the water need, which in turn influences the panicle numbers per unit area. Sustaining an elevated RLWC during this stage in the

colonized plants led to increased productive tillers, whereby 73.88% improvement in productive tillers and an 18.80% increase in filled grains per panicle were observed in the colonized plants under severe moisture stress (MS_5) compared to non-inoculated plants facing the same stress (Fig. 2). We earlier reported higher phosphorus uptake and enhanced root volume in *P. indica* colonized rice plants (18), which would have provided greater absorption area for water uptake and maintained plant water status, which in turn might have helped to keep higher RLWC even under stressful situations, alleviating the moisture stress in the plants. Hosseini *et al.* (31) also made similar observations in wheat.

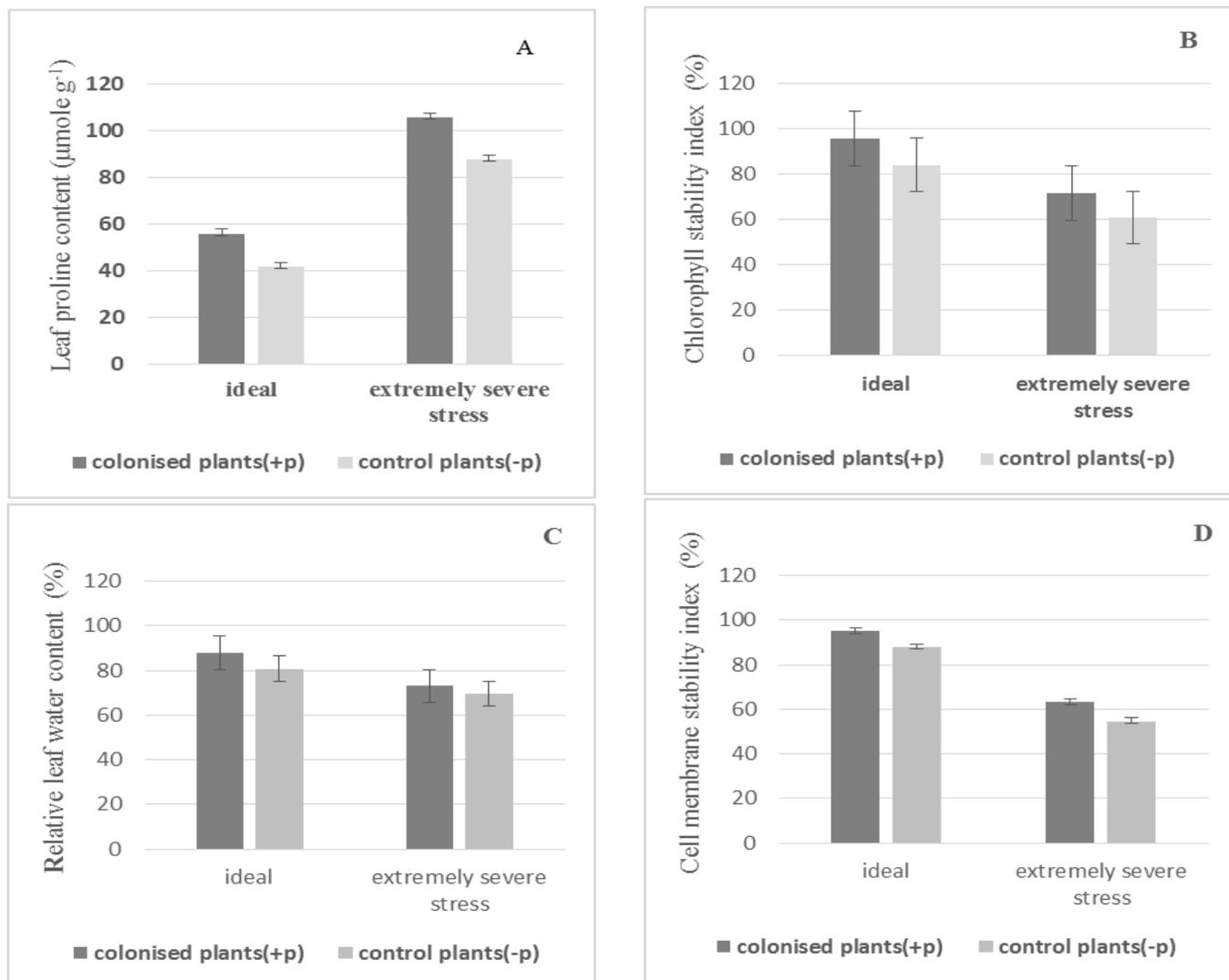


Fig. 1. Leaf proline content (A), Chlorophyll stability index (B), Relative leaf water content (C), and Cell membrane stability index (D), of rice plants grown with or without moisture stress. Mean values of the parameters from samples (n=15) from all the treatment combinations were analysed and compared, and only data from plants without stress and those with extremely severe stress are presented in the figure (mean \pm SD). Black bars represent plants with *Piriformospora indica* root colonization, and the grey ones without *P. indica* colonization.

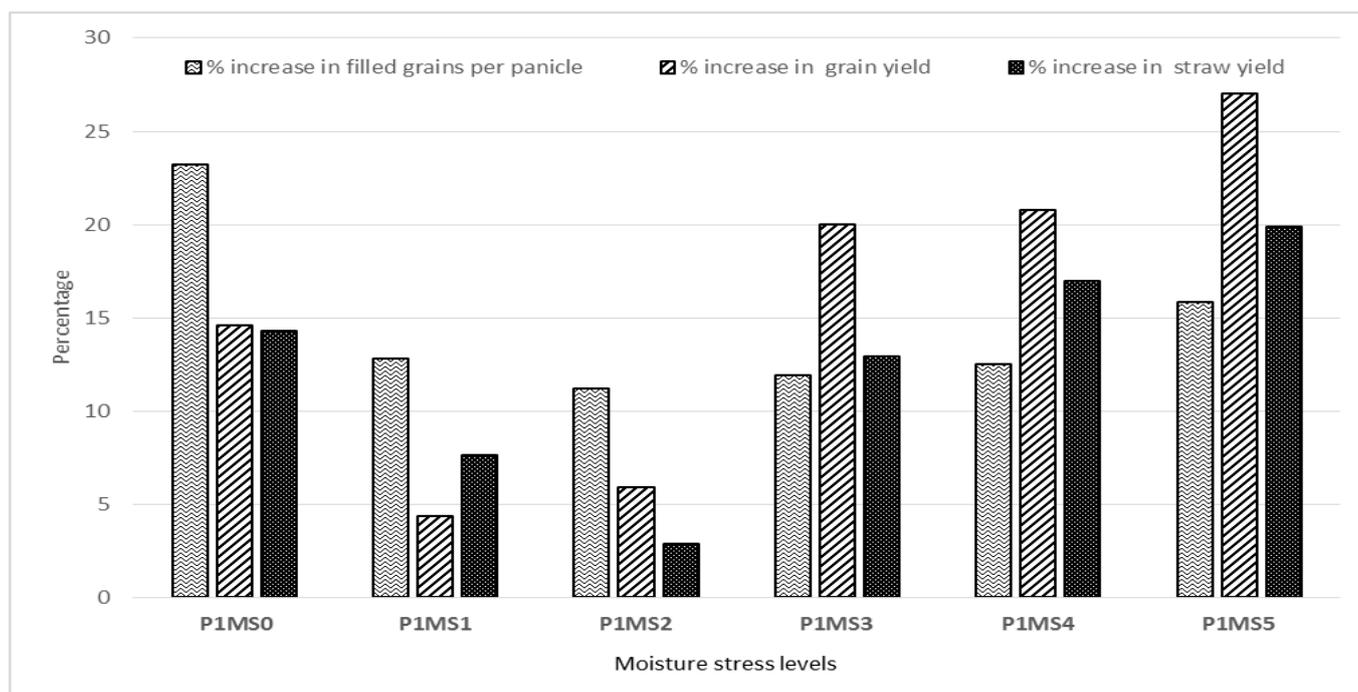


Fig. 2. Comparison of yield attributes of colonized rice plants with their non-colonized counterparts at different moisture stress levels.

P₁MS₀: colonised plants under no stress; **P₁MS₁**: colonised plants with mild stress; **P₁MS₂**: colonised plants under moderate stress; **P₁MS₃**: colonised plants under severe stress; **P₁MS₄**: colonised plants under very severe stress; **P₁MS₅**: colonised plants under extremely severe stress

Cell membrane stability defines the resistance of cell membrane against damage that restricts electrolyte leakage due to drought stress. Colonized plants irrigated to ideal conditions registered higher CMS (95 and 85%) at PI and flowering. The endophytic fungus facilitated adequate water uptake and higher acquisition of essential nutrients. Colonized plants under stress (irrigated at 40 mm CPE to a depth of 1.5 cm) recorded superior CMS (56.83 and 47.50%) at PI and flowering. It was 29.45 and 8.2% higher than non-inoculated stressed plants. At extremely severe stress, non-colonized plants had CMS of 88.10 and 78.76% at PI and flowering, respectively, which were 7.83 and 7.92% lower than those registered in colonized plants at corresponding stages. High CMS recorded in the present study can be correlated with the improved potassium uptake by the plant in the presence of fungus, leading to the maintenance of cell turgor. Premachandra *et al.* (32) observed that potassium nutrition improved cell thickness, leading to enhanced cell membrane stability during drought stress.

One of the major requisites for drought tolerance is the chlorophyll stability index, which specifies the stability of chlorophyll apparatus without damage or stress. Plants colonized by *P. indica* exhibited better chlorophyll stability under stressful conditions compared to non-colonized plants facing the same level of stress. This suggests that colonized plants have the ability to withstand the adverse effects of drought stress. The inoculated plants under extremely severe stress recorded 22.29 and 13.66% higher CSI than non-inoculated plants at PI and flowering (Fig. 1). It would be due to carotenoids production by *P. indica* that protected the chlorophyll apparatus under drought stress, resulting in high chlorophyll stability and ultimately high chlorophyll content under stress, enabling drought tolerance (33). At the same irrigation interval and depth, colonized plants recorded CSI of 66.2 and 49.9% at PI and flowering, which was 22.29 and 13.66% higher than non-colonized plants under extremely severe stress situations. Non-colonized plants recorded lower stability of chlorophyll apparatus, which might be due to the production of ROS in higher quantities under stressful situation (31). Enhanced CSI upon colonization lead to a 37.03% improvement in grain yield and straw yield saw a 12.71% increase compared to non-colonized plants (Fig. 2). Colonization positively influenced the CSI in terms of photosynthesis rates, leading to increased assimilate accumulation and translocation, resulting in a higher number of panicles and filled grains. Ghabooli *et al.* (34) noticed that *P. indica* colonization improved protein production necessary for photosynthesis, antioxidant defense system and energy transport, which might be a reason for high drought tolerance in colonized plants.

Conclusion

The co-cultivation of *Piriformospora indica* with rice has demonstrated a pronounced effect on the physiological and biochemical mechanisms, enhancing plant tolerance to drought stress. Notably, colonized plants exhibited elevated proline content under extremely severe stress

linked to osmotic adjustment, signifying the role of the endophyte in mitigating drought stress. Relative leaf water content, a crucial indicator of water status, was notably higher in colonized plants even under severe stress, indicating improved adaptability and hydration levels. Furthermore, colonized plants exhibited higher cell membrane stability, attributed to increased potassium uptake and maintenance of cell turgor, contributing to enhanced drought tolerance. The chlorophyll stability index showed significant improvement in colonized plants, showcasing the protective role of *P. indica* in maintaining chlorophyll apparatus stability and overall drought tolerance. Improved physiological factors due to colonization could enhance drought tolerance by preventing cell membrane and chlorophyll apparatus breakdown, fostering sustained photosynthesis, enabling the accumulation and translocation of assimilates to grains, leading to a higher yield with increased productive tillers and filled grains per panicle. The findings underscored the multifaceted contributions of *P. indica* endophytism in fortifying plants against the adverse effects of drought stress, offering valuable insights for sustainable agricultural practices.

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Authors' contributions

KMM performed the experiments and AM supervised the study. AS and SS arranged the resources. PPG performed statistical analysis. KMM and AM wrote the manuscript. KNA reviewed and edited the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no competing interests.

Ethical issues: None

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