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Effect of ACC deaminase producing bacteria on germination and seedling growth of rice under heat stress

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Abstract

Ethylene is a gaseous phytohormone regulating plant growth at all stages commencing from seed germination and development and extending to senescence. It is also a stress responsive hormone regulating responses of plants to abiotic and biotic stress conditions. The hormone has been associated with stress-induced senescence in plants and manipulation of ethylene synthesis is known to affect plant stress tolerance. 1-aminocyclopropane-1-carboxylate (ACC) deaminase is a bacterial enzyme that has been known to influence plant ethylene production by degrading the immediate precursor of ethylene biosynthesis specifically ACC, into ketobutyrate and ammonia resulting in reduced ethylene production during stress. In the present study, an ACC deaminase producing bacteria isolated from rhizosphere of rice and identified as *Rhizobium* sp was able to show ACC deaminase activity of 2.52 ± 0.73µM α- ketobutyrate /µg protein/24h. The bacterium was observed to partially offset the negative effects on seedling growth which occurred due to the presence of 3mM ACC in the growth medium. Rice seeds treated with *Rhizobium sp* showed highest germination percentage and vigour index under heat stress at 45 °C, when compared to uninoculated control.

Keywords: ACC, rice, *Rhizobium sp*, heat stress and germination

Introduction

The present day agriculture is plagued by various abiotic (extreme temperatures, drought, salinity, water logging) and biotic stresses (weeds, insect pests, nematodes and pathogens) affecting agricultural productivity (Mariani and Ferrante, 2017; Gimenez *et al*., 2018). Among the various stressors, heat and drought are most important, having a huge impact on growth and productivity of the crops (Fahad *et al.*, 2017). Rice *(Oryza sativa* L.), a major cereal crop and staple food for nearly half of the world population is highly vulnerable to high temperatures. With each 1 ºC rise in day temperature from 28 to 34 °C, a 7-8% reduction in rice yield has been predicted (Baker *et al*., 1992). Although high temperature affects all growth stages of rice from seed germination to seed setting and ripening (Shah *et al*., 2011), it's influence on seed germination and early seedling growth stage is crucial as these stages are important for obtaining a good plant stand and subsequently, high yields (Weitbrecht *et al*., 2011; Hasanuzzaman *et al*., 2013). The optimum temperature for germination of rice seed is 28- 30 °C while the threshold temperature at the seedling stage has been identified as 35 °C (Sarsu, 2018). Seedlings experience a decrease in stomatal conductance and photosynthetic rate due to high temperatures which can lead to poor plant growth (Sanchez-Reinoso *et al*., 2014; Yoshida, 1981)..

In response to stresses, plants protect themselves by modulating the expression of hormones which induce production of stress related proteins and other molecules. One important plant hormone that mediates stress response is ethylene, a gaseous phyto hormone which if accumulated in excess of threshold level, hinders plant growth and development. Manipulation of ethylene biosynthesis or perception has been advocated as a means to create plants with desirable traits like tolerance to various stresses (Stearns and Glick, 2003). Crop engineering targeting plant ethylene signaling as a new strategy for crop improvement, has resulted in crops that show improved growth in the field due optimization of their ethylene responses (Dubois *et al*., 2018).

Plant associated microbes also play a key role in maintaining plant health under various stresses (Kumar and Verma, 2018; Ma *et al*., 2019). The lowering of

ethylene levels is in fact considered as one of the major mechanisms employed by plant growth-promoting bacteria to sustain plant growth under stress. Certain bacteria produce an enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase by which the bacterium cleaves ACC, the precursor of plant ethylene biosynthesis into ketobutyrate and ammonia. Since ethylene production mainly depends on the endogenous levels of plant ACC (Gupta *et al*., 2019), these bacteria when associated with plants acts as a sink for ACC, thereby decreasing internal ACC levels leading to a concomitant reduction in plant ethylene production (Glick, 2014). Inoculation of plants with bacteria expressing ACC deaminase activity has been helpful in allowing plant growth and development under stress conditions by reducing stress-induced ethylene production and significantly decreasing the severity of stresses (Saleem *et al*., 2007; Glick, 2014).

In the present investigation, an ACC deaminase producing plant growth promoting bacteria isolated from rice rhizosphere was evaluated for its ability to attenuate the effect of exogenously supplied ACC and to improve rice seed germination and seedling growth under heat stress.

Materials and Methods

Rhizobium sp. with accession number (KY348774) maintained at ICAR- Indian Institute of Rice Research, Hyderabad was used for the study. Seeds of heat susceptible genotype, *O. sativa* cv. Swarna were also obtained from ICAR-IIRR.

Quantification of ACC deaminase activity

Rhizobium sp. was grown overnight in nutrient broth, centrifuged at 10,000 rpm for 10 min and the cell pellet obtained was washed with modified Dworkin and Foster minimal medium (DF) (containing g/l KH_2PO_4- 4g, Na_2HPO_4 - 6g, $MgSO_4.7H_2O$- 0.2g, glucose - 2g, gluconic acid- 2g, citric acid- 2g, trace elements ($FeSO_4.7H_2O$- 1mg, H_3BO_3- 10mg, $MnSO_4.7H_2O$- 11.19mg, $ZnSO_4.7H_2O$- 124.6mg, $CuSO_4.5H_2O$- 78.22mg, MoO_3- 10mg). After washing, the cell pellets were suspended in DF minimal broth containing 3mM ACC (1- amino cyclopropane carboxylic acid), incubated for 24 h, washed in Tris buffer (pH- 7) and resuspended again in Tris buffer (pH-8.5) and toluene. Toluenized cell suspensions were used for measuring ACC deaminase activity according to the procedure of Dworkin and Foster (1958). The ACC deaminase activity was estimated spectrophotometrically as α-ketobutyrate production at 540 nm using a standard curve of α-ketobutyrate ranging between 1- 10µM concentrations (Honma and Shimomura, 1978). Protein content in the toluenized cell suspension was determined by Lowry's method with bovine serum albumin (200-1000 µg/ml) serving as standard (Lowry *et al*., 1951). ACC deaminase activity was expressed as the amount of α-ketobutyrate liberated in nmol per milligram of cellular protein per 24 h.

PCR amplification of ACC deaminase producing (*acdS)* gene

ACC deaminase (*acdS)* gene was amplified from the DNA extracted from the isolate using ACCf (5´ATGAACCTGAATCGTTTTRAA 3´) as forward primer and ACCr (5´TCAGCCGTTGCGRAACARAACARGAA3´) as reverse primer. PCR was performed in a Thermal Cycler (T-100, Biorad) with the following conditions for 35 cycles, *i.e.,* initial denaturation at 95 °C for 5 min, initial extension 72 °C for 1 min and annealing temperature at 55 °C for 45 sec followed by final denaturation at 95 °C for 1min and final extension 72 °C for 10 min, with expected PCR product being ~996 bp (Farajzadeh *et al.,* 2010).

Seedling survival under exogenous ACC treatment

Three day old seedlings of rice (cv. Swarna) grown under aseptic conditions were transferred to sterile test tubes containing 10 ml of Yoshida medium (containing g/lit: NH_4NO_3- 91.4, NaH_2PO_4. $2H_2O$- 35.6, K_2SO_4- 71.4, $CaCl_2$. $2H_2O$- 117.35, $MgSO_4.7H_2O$- 324 and micronutrients: $MnCl_3.2H_2O$-1.5, $(NH_4)6Mo_7O_{24}4H_2O$-0.074, $ZnS0_4.7H_20$-0.14, H_3BO_3- 0.934, $CuSO_4.5H_2O$- 0.031, $FeCl_3.6\ H_2O$- 7.7 and citric acid- 11.9) and seedlings were grown at 28 ± 30 °C under 16 h/8 h light and dark illumination regime in the presence and absence of 3mM ACC and *Rhizobium sp*. inoculation (1x 10^8 CFU/ml). Seedling growth was evaluated after ten days by measuring the root and shoot growth.

In vitro assay for heat stress tolerance

Seed germination and seedling growth of rice seeds treated with *Rhizobium* sp were evaluated after imposition of heat stress. Surface sterilized Swarna seeds were soaked overnight in bacterial suspension (3 x 10^8 CFU/ml of *Rhizobium sp.*) and allowed to germinate at, i) ambient temperature of 30 °C while a second set of seeds, ii) were exposed to 45 °C heat stress for 24 h (Mastouri *et al*., 2010) and then grown at ambient temperature. Rice seeds soaked in sterile water were used as control. After seven days, the lengths of root and shoots of germinating seeds were

recorded and used for calculation of germination indices (Kandasamy *et al*., 2009)

Germination percentage (%) = (Number of seeds germinated in petri plate/Total no of seeds in the petri plate used for test) X 100

Vigour Index = (Mean of root + shoot length) x Germination percentage

Vigour Index Increment = Vigour index in treatment – Vigour index in control

Statistical analysis

The experiments were conducted in completely randomized design with three replicates and the data are presented as mean ± SD.

Results and Discussion

ACC deaminase production

ACC is taken up by bacterial cells and the ACC deaminase enzyme found inside the cytoplasm of bacterial cells degrades ACC into α-ketobutyrate and ammonia. The bacterial isolate used in this study, identified as *Rhizobium sp.* showed ACC deaminase activity of 2.52 ± 0.73 µM α-ketobutyric acid /µg protein/24h (Table 1).

Table.1 ACC deaminase activity of the bacterial isolate

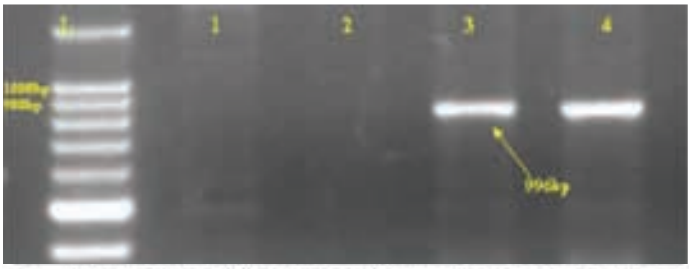
S.No	Name of the isolate	ACCdeaminase activity* µM α – ketobutyrate /µg protein/24h
1	*Rhizobium sp.*	2.52 ± 0.73

*Mean ± SD of three replicates

According to Glick (2005), high ACC deaminase activity is observed in rhizosphere, phyllosphere and endophytic bacterial inhabitants and the bacteria present in these niches can act as a sink for ACC produced by plant as a consequence of stress. Studies by Timmusk (2011) have also revealed that ACC deaminase activity is relatively common in rhizosphere bacteria, especially in soils that are often subjected to stressful conditions. Rice rhizosphere bacteria exhibiting ACC deaminase activity was also observed by Bal *et al*., (2013). ACC deaminase enzyme has been found in both Gram negative and Gram-positive bacteria and also some fungi (Saleem *et al*., 2007). The presence of ACC deaminase activity in *Rhizobium* was also reported by Duan and his colleagues (2009) in a study wherein 27 strains of *Rhizobium* (12%) expressed ACC deaminase during a screening of large number (233) of rhizobial isolates.

ACC deaminase gene amplification

Growth on ACC medium and a detectable deaminase activity in general is not considered confirmative of bacteria with ACC deaminase enzyme production. The reasons attributed are that nitrogen-fixing bacteria without ACC deaminase activity are able to grow on the ACC medium, while certain other bacteria are able to grow on trace amounts of nitrogen present in medium components and in the agar used for isolations (Li *et al*., 2015). Hence, the presence of ACC deaminase structure gene (*acdS*) is important for predicting and for identifying ACC deaminase production by bacteria. PCR amplification of the *acdS* gene using degenerate primers has been widely employed for confirmation of ACC deaminase activity. *Rhizobium* sp used in this investigation showed ACC deaminase gene amplification with the expected product size of 996 base pairs using gene specific primers (Figure1) as described by (Farajzadeh *et al*., 2010; Mahmooda *et al*., 2019) thereby confirming the ACC deaminase activity of the bacterium.



L – 100bp Ladder, 1. Water blank, 2. *Gluconacetobacter diazotrophicus*, 3. *Serratia marcescens* and 4. *Rhizobium* sp.

Figure 1. Amplification of *acds* gene (996 bp) from *Rhizobium sp.*

Effect of exogenous ACC application and bacterial inoculation on seedling growth

Exogenously applied ACC is known to increase ethylene production in plant tissues (Shaharoona *et al*., 2007) including rice. Dark-grown seedlings of rice show a double response in differential root and shoot growth due to ethylene treatment (Ma *et al*., 2013). In this study, rice seedling grown in the presence of ethylene precursor ACC showed comparatively reduced root and shoot growth (5.1 and 9.1 cm respectively) relative to untreated seedlings (3.8 and 5.3 cm respectively). Rice seedlings which were inoculated with the isolated *Rhizobium* strain was found to improve both root and shoot growth irrespective of the presence of ethylene precursor in the growth medium when compared to uninoculated seedlings. Average root growth of 6.7 cm and shoot growth of 12.8 cm were observed

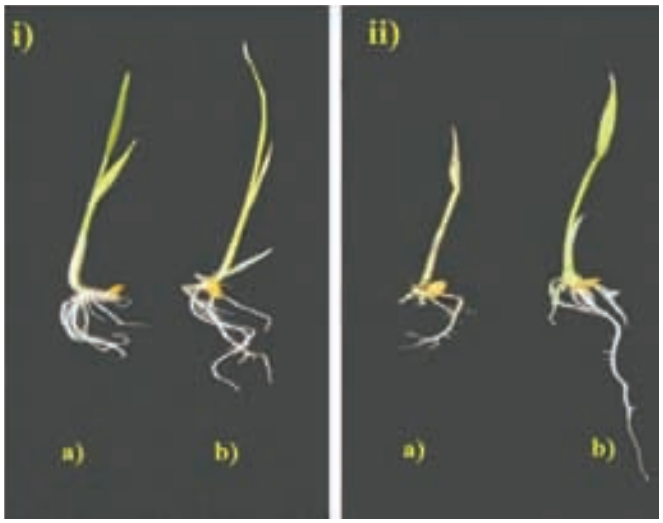
in rice seedlings grown in the presence of the isolated *Rhizobium* strain (Table 2).

Table 2. Rice seedling response to ACC and *Rhizobium sp.* inoculation

S. No	Treatment	Root length (cm)*	Shoot length (cm)*
1	Control	5.1 ± 0.55	9.1 ± 0.55
2	*Rhizobium sp.*	6.7 ± 0.17	11.3 ± 0.2
3	Control + 3mM ACC	3.8 ± 0.7	5.3 ± 1.1
4	*Rhizobium sp.* + 3mM ACC	5.4 ± 0.25	6.7 ± 0.35

*Mean ± SD of three replicates

The reduction in growth due to ACC treatment was partly alleviated by the presence of inoculated bacteria as rice seedlings grown in the presence of both ACC and *Rhizobium* showed better root and shoot growth (5.4 cm and 6.7 cm respectively) compared to seedlings treated with ACC in the absence of bacteria (Figure 2). The dilution of the effect of ACC on plant growth could probably be due deamination of ACC by the isolate into a-ketobutyrate and ammonia (Barnwal *et al*., 2014; Heydarian *et al*., 2016., Ali and Kim, 2018; Saleem *et al*., 2018; Zhang *et al*., 2018).



Seedling growth in i) absence and ii) presence of ACC under a) uninoculated and b) inoculated (*Rhizobium* sp.) conditions

Figure 2. Rice seedling growth in the presence of *Rhizobium sp.* and ACC

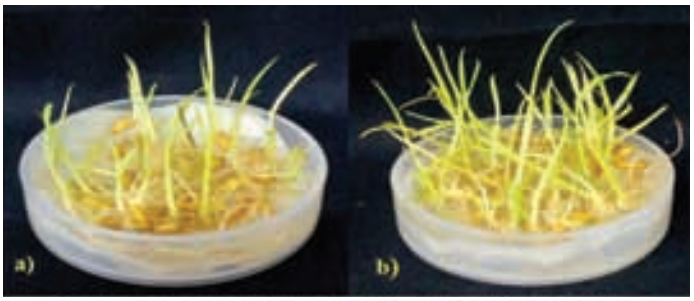
Effect of *Rhizobium* inoculation on germination and seedling growth under heat stress

Seeds inoculated with *Rhizobium* recorded increased germination percentage of 86 and 34% when compared to control (82 and 20%) under ambient and heat stressed conditions. Similarly bacterial treatment also increased vigour index (1074.1 ± 9.49 and 177.32 ± 39.44 under ambient and heat stress respectively), contrary to control uninoculated seedlings with vigour indices of 831.48 + 7.71 and 55.6±21.35 under ambient and heat stress respectively. Vigour index increment due to inoculation was 242.66 ± 23.4 and 121.72 ± 54.78 under ambient and heat stressed conditions (Table 3). Seed inoculation with the isolate was able show improved germination when compared to uninoculated control under ambient and heat stressed conditions (Figure 3).

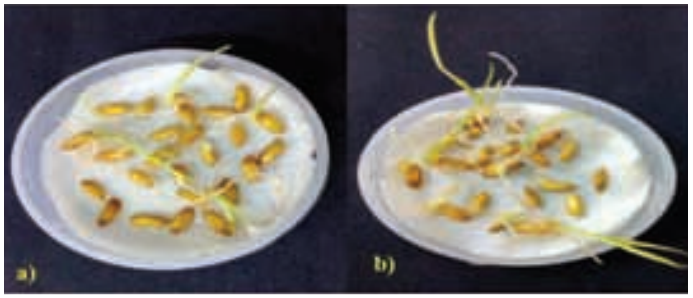
Table 3. Effect of *Rhizobium sp.* inoculation on rice germination indices under heat stress.

S. No	Germination indices	Control		*Rhizobium sp.*	
		Ambient	Temp (45 °C)	Ambient	Temp (45 °C)
1	Germination percentage*	82 ± 19.8	20 ± 10	86 ± 13.4	55 ± 39.43
2	Vigour index*	831.48 ± 7.71	55.6 ± 21.35	1074.1 ± 9.49	177.32 ± 39.44
3	Vigour index increment*	-	-	242.66 ± 23.4	121.72 ± 54.78

*Mean ± SD of three replicates



Germination in ambient conditions: a) control and b) + *Rhizobium sp.*



Germination under heat stress (45°C): a) control and b) + *Rhizobium sp.*

Figure 3. Effect of *Rhizobium sp.* inoculation on germination of rice under heat stress

Fluctuation in temperature leads to hormonal imbalances in plants with changes in ethylene phytohormone in particular leading to growth retardation. Microorganisms have been demonstrated to play a key role in alleviating the stress induced in plants caused due to abiotic and biotic

factors (Grover *et al*., 2011; Glick, 2014; Gamalero, 2015 and Gupta *et al*., 2019). Thermotolerance in sorghum seedlings was observed to be induced by *Pseudomonas* sp. that improved biochemical status of plants in terms of proline, sugar, amino acid and chlorophyll content (Ali *et al*., 2009) thereby leading to improved plant biomass. Seed treatment using another *Pseudomonas aeruginosa* strain 2CpS1 was found to ameliorate the deleterious effects of temperature stress on wheat (Meena *et al*., 2015). In concordance with our results, previous studies have also reported that ACC deaminase producing growth promoting bacterium *Burkholderia phytofirmans* PsJN could protect potato plants in maintaining normal growth under heat stress (Bensalim *et al*., 1998).

Conclusion

Plant-associated bacteria can play an important role in conferring tolerance in crops against abiotic stresses. In this study, a rice rhizospheric bacterium identified as *Rhizobium sp.* was able to impart heat stress tolerance to rice seedlings. Subsequent follow through experiments in glasshouse and field conditions can consequently lead to the use of these beneficial bacteria as a cost effective and environmental friendly approach for ensuring sustainable rice production under heat stress induced by high temperatures.

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