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Evaluation of *Pseudomonas* spp. and *E. cloacae* against *Fusarium oxysporum* in chickpea under Drought *in vivo* conditions

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ABSTRACT: Three rhizospere bacterial strains were collected from chickpea and three strains found to be potential against *Fusarium oxysporum* and identified through 16s rRNA sequencing as *Pseudomonas* spp, *E. cloacae* and *P. chlororaphis.* The present study was conducted during 2019-2020 and 2020-2021 at Department of Molecular Biology and Genetic Engineering, RTMNU, Nagpur, Maharashtra, to evaluate the effects of PGPR with biocontrol activity on biochemical parameters under *in vivo* conditions in chickpea KWR-108 genotype under drought. The pooled data of two experiment results recorded T7 (A+B) PGPR treatment with consortium of *Pseudomonas* spp. and *P. chlororaphis* ranged highest in chlorophyll 'a' with 25.75 (mg/g). In chlorophyll 'b' highest recorded with 44.82 (mg/g) in T7 (A+B), whereas in total chlorophyll content highest ranged between 10.86 (mg/g) in T8 (A+C) with consortium of *Pseudomonas* spp. and *E. cloacae.* In carotenoid content, the highest value reported in T7 (A+B) with 36.21 (µg/g) and high protein content ranged between 0.98 % in T7 (A+B). According to the results of the current reports, T7 (A+B) with consortium of *Pseudomonas* spp. and *P. chlororaphis* found to be best PGPR treatment with comparing to other treatments in both experiments against *Fusarium oxysporum* and for mitigating drought stress in KWR-108 variety of chickpea. Therefore this study related to PGPR treatments is helpful for the marginal farmers to overcome biotic and abiotic stress reported in this current investigation.

Keywords: Chickpea, *Fusarium oxysporum*, PGPR, drought, Biochemical.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is considered as major pulse crops not only in India but throughout the world and is the mostly important staple food in several leading countries. It ranks 5th among grain crops and is imperative due to its high nutritive contribution in Indian diet. It has considerable importance in fodder also (Montenegro *et al.,* 2010). Duirng in the year 2017-2018, chickpea was cultivated approximately 106 L ha in India alone, recorded a harvest production of >111 Lt as highest productivity level of 1056 kg-ha. Whereas in Maharashtra, terms of area is 18% (PRFFTNS 2018). However, productivity of chickpea in India is far behind as compared to that of world productivity.

Plants reported several non-biotic stresses, water deficit stress would unfavorably affect the growth and development in plants leading ultimately to morphological, physiological, molecular and biochemical changes **(**Potters *et al.,* 2007). Inorder to enhance the potentiality of photosynthetic machinery, a prokaryotic osmoregulatorycholine oxidase gene (codA) was targeted to overcome the negative effects caused by oxidative damage at the chloroplasts (Reddy *et al.,* 2012).

Production of chickpea is majorly affected by several biotic stresses globally, including, bacterial, fungal, viral diseases, pests, insects, parasitic weeds and nematodes. *Fusarium* Genus is a filamentous saprophytic fungus distributed well by affecting animal, health of human and plants, as they penetrate the food chain (Smith *et al.,* 1988). *F. oxysporum* leads to harsh wilting in chickpea genotypes within 25 days after sowing in field conditions which are highly susceptible. This pathogen penetrates and reaches the xylem through roots. Here, it either decreases or completely

blocks the aerial parts from transport of water that ultimately leads to death **(**Halila *et al.,* 2009).

The application of plant growth promoting rhizobacteria (PGPR) would be the alternative solution to overcome the negative effects and ability to cope up with various biotic and abiotic stress factors. This also aids the chickpea cultivation more independent on chemical fertilizers leading to sustainability **(**Sagar *et al.,* 2021). These microbes are interconnected with roots of the plant and are identified to be advantageous for the delayed leaf senescence, increased yield, germination rate, nutrient availability, leaf area, protein content, chlorophyll content and overall growth (Sagar *et al.,* 2020c). Several literatures have specified that PGPR efficiently mitigate the destructive impacts of several stresses through diverse mechanisms and act as plant helpers in combating stress by increasing the tolerance in plants (Laloo *et al.,* 2017). The objective of the present study is to study the effects of (Plant growth promoting rhizobactera) PGPR with biocontrol activity on biochemical parameters in situ (field) studies.

MATERIAL AND METHODS

The present investigations were carried out during 2019-2020 and 2020-2021 at Department of Molecular Biology and Genetic Engineering, Rashtrasant Tukdoji Maharaj Nagpur University (RTMNU), Nagpur, Maharashtra.

Chickpea Variety:

Chickpea variety KWR 108 was released in the year 1996 by Chandrashekar Azad University of Agriculture & Technology (CSAUAT), Kanpur and seeds were obtained from ICAR-IIPR, Kanpur. The silent features are: seeds are dark brown and small and resistant to wilt, 130-135 days to maturity and production 20-23Quintal/Hectare.

PGPRs Details:

Three PGPRs were obtain culture collection of Department of Biological Science SHUATS Prayagraj.

Pathogen Details:

Pathogen- *Fusarium Oxysporum* Obtain from Department of Molecular and Genetic Engineering (Rashtrasant Tukdoji Maharaj Nagpur University) RTMNU, Nagpur, Maharashtra.

Seed treatment:

Seeds were priorly soaked overnight by three PGPRs with single and consortium treatments according to compatibility test results.

Pot and Field trials: Experiments were carried out during 2019-2020 (Polyhouse conditions) and 2020-2021 at research fields according to RBD layout, Department of Molecular Biology and Genetic Engineering, Rashtrasant Tukdoji Maharaj Nagpur University (RTMNU), Nagpur, Maharashtra.

Plate 1: Preparation of field trial and soil inoculation with *F. oxysporum.*

Plate 2: Pot trail experiment conducted at polyhouse of RTMNU.

Plate 3: Research field work at Department of Molecular Biology and Genetic Engineering, RTMNU.

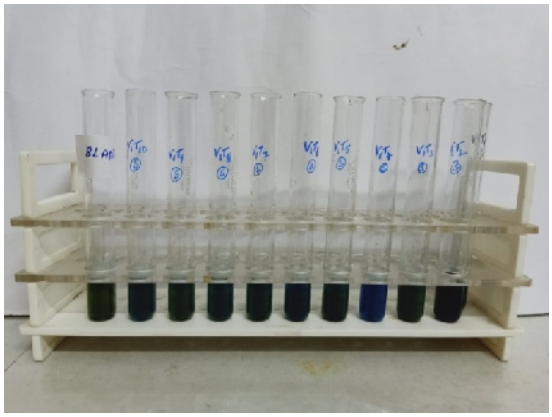

Plate 4: Estimation of protein content in leaves of chickpea.

Treatment details:
T1- control
T2- control (pathogen-*Fusarium oxysporum*)
T3- control (with *drought*)
T4- Isolate A (*Pseudomonas* spp.)
T5- Isolate B (*P. chloraphis*)
T6- Isolate C (*E. cloacea*)
T7- Isolate A+B
T8- Isolate A+C
T9- Isolate B+C
T10- Isolate A+B+C

Dual culture test: Bacteria inoculated on both the edges remaining bacteria inoculated as centre, as the control plate of pathogen was recorded 90 mm diameter petri plate with pH 6.1**,** incubated at 30°C on PDA medium. After 24 h, a 5-7 mm of diameter active fungus was inoculated at the center with a 12-h photoperiod. The zone of inhibition in between the 2 bacterial cultures was measured on following 72 hours after inoculation (Krishna *et al.*, 2005).

Chlorophyll content: Chlorophyll was observed by obtained 1 gm of leaf samples at maturity phase starting, and these were weighed again and crushed with 80% of acetone to it and made up to volume by 20ml. these were centrifuged for 10 minutes at 600 rpm. The supernatant collected in a fresh tubes for the spectrophotometer observations at 663nm and 645nm and calculated according to the given below formulas for chlorophyll 'a', 'b' and total chlorophyll content (Wellborn, 1983).

$$\text{Chlorophyll'a'}(mgg-1F.W.) = 12.7\,x\,(A663) - 2.69\,x(A645)x\frac{V}{1000\,xwxa}$$

$$\text{Chlorophyll'b'}(mgg-1F.W.) = 22.9\,x\,(A645) - 4.68\,x\,(A663)\,x\frac{V}{1000\,xwxa}$$

$$\text{Total chlorophyll}\,(mgg-1F.W.) = 20.2\,x\,(A645) + 8.02\,x\,(A663)\,x\frac{V}{1000\,xwxa}$$

Carotenoid content: Carotenoid was determined according to **(**Wellborn, 1983). 1 gram leaves sample weighed and crushed with 80% acetone made up the volume to 25 ml with 80% acetone and the centrifugation was at 3000 rpm at 10 min. Supernatant collected was observed readings at 470nm spectrophotometer and calculated accordingly by given formula.

$$\text{Total carotenoids} = \frac{[1000\,A470 - (3.27\,Chla + 104\,Chlb)]}{229}$$

Protein: it was determined by collected 0.5gm of leaf sample from every treatment crushed with phosphate buffer in a mortar and pestle. This was centrifuged and collected into another new test tube at -20°C for an hour or two, as centrifugation was done for 10-15 minutes at 12000rpm at 4°C in every bradford reagent 2 ml, 10 µl (sample) in 990 µl (distilled H_2O) was mixed, and measured at 595nm, hence calculated by bovine serum (Bradford, 1976).

Statistical Analysis. The statistical analysed for the data obtained in this current investigation was by two way factor by OPSTAT software RBD (randomized block design). It was correlated to analysis of variance at 5% level and was found to be significant. The pooled was done analyses as per **(**Gomez and Gomez, 1984).

RESULTS

The further investigation was done under *in situ* conditions in single and consortium treatments of above said three PGPR isolates according to compatibility test results.

Biocontrol activity: Thirty one isolates were screened under *in vitro* from chickpea rhizosphere to perform dual culture technique for biocontrol and antagonistic activities. Three isolates mentioned in this study were found to be potential with high antagonism activity. PR1 identified as *E. cloacae* reported high inhibition of 62.21%, followed by PR21 identified as *P.* spp. with high inhibition of 61.85%, nextly by isolate PR29 identified as *P. chlororaphis* with 55.36% of inhibition against *F.o.c.* during dual culture assay, where control was maintained at 90mm in petriplate. Inhibition zone in mm was recorded and inhibition percentage was calculated accordingly, the results thus obtained were shown in Fig. 1.

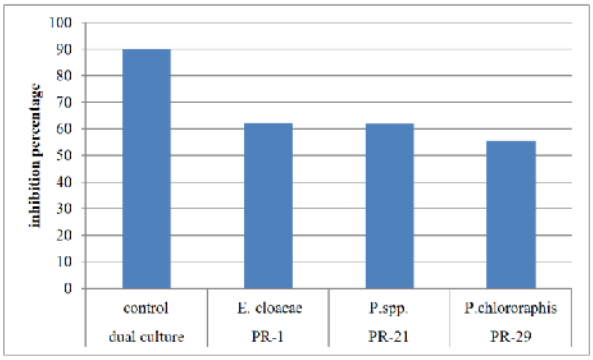

Fig. 1. Variation of Graphical representation in inhibition percentage of dual culture assay, against *F.o.c.*by potential PGPR isolates under *in vitro* conditions.

Chlorophyll 'a' (mg/g): In variety KWR-108, the differential response for the chlorophyll 'a' under single and combined PGPR treatments were recorded highest as 25.52 mg/gin T9 (B+C) with consortium (*E. cloacae*

and *P. chlororaphis)* and 32.94 mg/gin T7 (A+B) (*Pseudomonas* spp. and *P. chlororaphis)* in contrast to other treatments against *Fusarium oxysporum* during 2019-2020 and 2020-2021 (Fig. 2). Moreover the maximum chlorophyll 'a' was observed as 25.79 mg/gin T7 (A+B) according to the pooled data. Simultaneously the minimum chlorophyll 'a' recorded in T3 as 14.21 mg/g followed by 4.37 mg/gin two consecutive years. However the least was recorded as 9.29 mg/g in pooled data of both the cropping years in T3 as pathogen treated plants over untreated and drought controlled treatments.

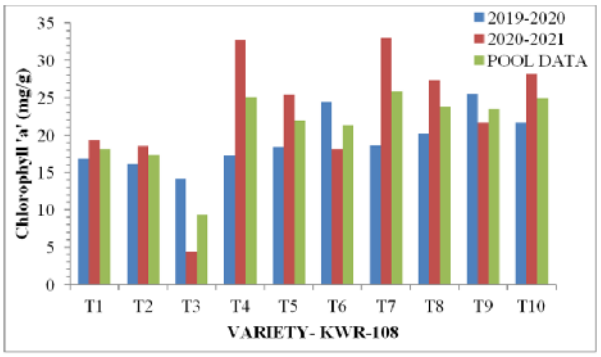

Fig. 2. Graphical representation of variation in chlorophyll 'a' (mg/g) in KWR-108 variety due to potential PGPRs against *Fusarium oxysporum* in drought stress management within two consecutive growing seasons (2019-20 and 2020-21) and pool data.

Chlorophyll 'b' (mg/g): In variety KWR-108, the differential response for the chlorophyll 'b' under single and combined PGPR treatments were recorded highest as 46.17 mg/gin T6 (B) with single treatment of *E. cloacae* and 59.93 mg/gin T7 (A+B) (*Pseudomonas* spp. and *P. chlororaphis)* in contrast to other treatments against *Fusarium oxysporum* during 2019-2020 and 2020-2021 (shown in Fig. 3).

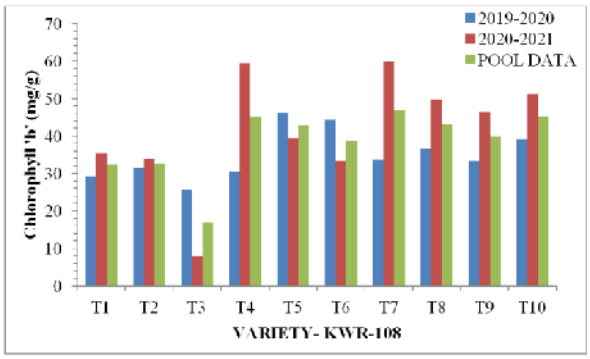

Fig. 3. Graphical representation of variation in chlorophyll 'b' (mg/g) in KWR-108 variety due to potential PGPRs against *Fusarium oxysporum* in drought stress management within two consecutive growing seasons (2019-20 and 2020-21) and pool data.

Moreover the maximum chlorophyll 'b' was observed as 44.82 mg/gin T7 (A+B) according to the pooled data. Simultaneously the minimum chlorophyll 'b' recorded in T3 as 25.72 mg/g followed by 8.1 mg/gin two consecutive years. However the least was recorded as 16.92 mg/g in pooled data of both the cropping years in T3 as pathogen treated plants over untreated and drought controlled treatments.

Total chlorophyll content (mg/g): In variety KWR-108, the differential response for the total chlorophyll content under single and combined PGPR treatments were recorded highest as 51.04 mg/g in T8 (A+C) with consortium of (*Pseudomonas* spp. and *E. cloacae)* and 90.35 mg/gin T4 (A) (*Pseudomonas* spp.) in contrast to other treatments against *Fusarium oxysporum* during 2019-2020 and 2020-2021 (Fig. 4). Moreover the maximum total chlorophyll content was observed as 70.86 mg/gin T8 (A+C) according to the pooled data. Simultaneously the minimum total chlorophyll content recorded in T3 as 28.47 mg/g followed by 23.28 mg/g in two consecutive years. However the least was recorded as 25.88 mg/g in pooled data of both the cropping years in T3 as pathogen treated plants over untreated and drought controlled treatments.

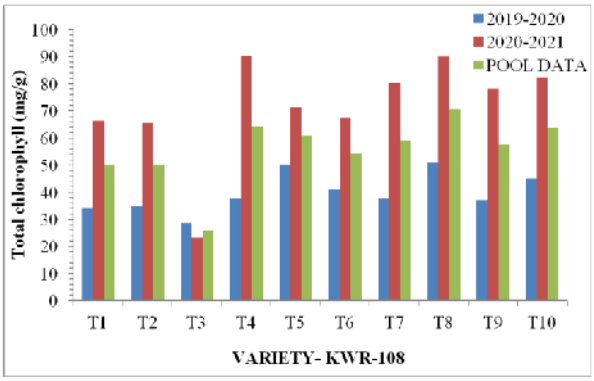

Fig. 4. Graphical representation of variation in total chlorophyll (mg/g) in KWR-108 variety due to potential PGPRs against *Fusarium oxysporum* in drought stress management within two consecutive growing seasons (2019-20 and 2020-21) and pool data.

Carotenoid content (µg/g): In variety KWR-108, the differential response for the carotenoid content under single and combined PGPR treatments were recorded highest as 24.59 µg/gand 47.84 µg/g in T7 (A+B) with consortium of (*Pseudomonas* spp. and *P. chlororaphis)* in contrast to other treatments against *Fusarium oxysporum* during 2019-2020 and 2020-2021 (shown in Fig. 5). Moreover the maximum carotenoid content was observed as 36.21 µg/g in T7 (A+B) according to the pooled data. Simultaneously the minimum carotenoid content recorded in T3 as 14.37 µg/g followed by 32.67 µg/g in two consecutive years. However the least was recorded as 23.52 µg/g in pooled data of both the cropping years in T3 as pathogen treated plants over untreated and drought controlled treatments.

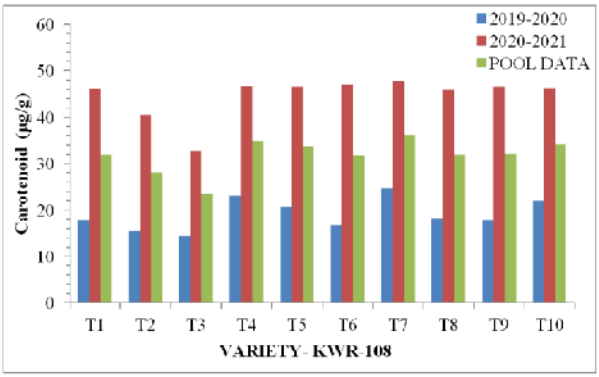

Fig. 5. Graphical representation of variation in carotenoid (µg/g) in KWR-108 variety due to potential PGPRs against *Fusarium oxysporum* in drought stress management within two consecutive growing seasons (2019-20 and 2020-21) and pool data.

Protein content (%): In variety KWR-108, the differential response for the protein content under single and combined PGPR treatments were recorded highest as 0.15 (%)and 1.8 (%) in T7 (A+B) with consortium of (*Pseudomonas* spp. and *P. chlororaphis*) in contrast to other treatments against *Fusarium oxysporum* during 2019-2020 and 2020-2021 (shown in Fig. 6). Moreover the maximum protein content was observed as 0.98 (%) in T7 (A+B) according to the pooled data. Simultaneously the minimum protein content recorded in T3 as 0.11 (%) followed by 0.27 (%) in two consecutive years. However the least was recorded as 0.19 (%) in pooled data of both the cropping years in T3 as pathogen treated plants over untreated and drought controlled treatments.

Fig. 6. Graphical representation of variation in protein (%) in KWR-108 variety due to potential PGPRs against *Fusarium oxysporum* in drought stress management within two consecutive growing seasons (2019-20 and 2020-21) and pool data.

DISCUSSION

PGPR ameliorated the synergistic effects of water deficit stress by improving osmoregulation and water balance by declining the chlorophyll, protein degradation and can maintain healthy photosystem too which effectively decline ROS damage (Khayatnezhad *et al.,* 2011). The chlorophyll content in this present study resulted to be high in comparison to un treated controlled treatments under drought effects when inoculated with PGPR *Pseudomonas* spp. treatment (Zaeifizade *et al*., 2009).

The metabolite level alters and increases potential PGPR performance with *Pseudomonas* spp. consortium by producing higher sugar, protein content compared to controlled treatments and provide undamaged photosystem in chickpea. Increase in carotenoid and chlorophyll content in PGPR treated plants supported by many literatures could be attributed to advanced accessibility of organic matter and nutrients in rhizosphere of chickpea (Esitken *et al.,* 2006).

During drought, chlorophyll is damaged due to excessive production of ROS that results in destruction of cell organelles and cellular metabolism in leaf tissue **(**Abbas *et al.,* 2013). The water deficit stress conditions eventually declines photosynthetic activities which finally leads to delayed photosynthetic pigments into photosystem complexes and its synthesis (Rani *et al.,* 2008).

The chlorophyll ‘a’, ‘b’ and total chlorophyll content was recorded to be increased significantly, resulting from the PGPR treatment with *Pseudomonas* spp. with respect to control, under stress condition in chickpea. The concerted action of both antioxidants like non-enzymatic and enzymatic system alleviates oxidative damage in plants such as ascorbic acid, β-carotenes and enzymes like superoxide dismutase, peroxidase, catalase and glutathione reductase (Gaurav *et al*., 2016). Carotenes plays as antioxidant defense system but they are very susceptible to oxidative damage caused due to water stress conditions, although several reports depicted that PGPR inoculation with *Pseudomonas* spp. accelerate the photosynthetic pigments. This helps from photo damage and facilitate the harmless dissipation of excitation energy to light collecting chlorophyll antenna, thus protecting chlorophyll (Prochazkova *et al.,* 2001).

The β-carotene in the chloroplasts bound to the core complexes of PS- I and PS-II that provides protection against ROS harmfull effects, through direct quenching of triplet chlorophyll which prevents the singlet oxygen generation and protects from oxidative damage in this current study reports. β-carotene not only acts as effective antioxidant but also plays vital role in sustaining and protecting photochemical process as an accessory pigment (Wahid, 2007).

One of the common consequences during stress is protein damage, so maintaining protein content in leaves is necessary for plant survival. Significantly, leaf protein content increment was evident after treated with PGPRs single and consortium in this current study with *Pseudomonas* spp. in comparison to untreated when grown under drought conditions in legume plants **(**Afzal *et al.,* 2008). This PGPR helps to mitigate ROS

over production and to synthesize antioxidant enzymes, heat shock proteins and other harmones, in order to cope with environmental stresses (Wani *et al.*, 2016). Proteins levels decreases in leaves during drought due to the declined synthesis. The present investigation has been supported by many literatures considering *Pseudomonas* spp. altered gene expression at water stress Shown in Plate 4, therefore mRNAs and new proteins were synthesis, helped to withstand during drought against *F. oxysporum.* In addition, these involved in signal transduction regulation and gene expression and transcription factors (Seyed *et al.*, 2014).

CONCLUSION

From the results, it is concluded that T7 (A+B) is the best combination treatment comprising of *Pseudomonas* spp. and *Pseudomonas chlororaphis* in control of *Fusarium oxysporum* and mitigating drought stress in KWR-108 variety of chickpea in terms of biochemical parameters such as chlorophyll, caratenoid and protein content. Therefore PGPR would be suitable reason to overcome reported pathogen even under water deficit conditions.

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Conflict of Interest. None.

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