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Multifaceted impact of *Bradyrhizobium sp*. bacteria in enhancing the growth and resilience of *Vigna mungo* genotypes under fluoride stress

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ABSTRACT

Agricultural produce has been declining owing to variety of factors, fluoride being one of them is known for its phytotoxic effects. Thus, this study was carried out to investigate the potential of a N_2 fixing bacteria, specifically *Bradyrhizobium sp*., in providing relief against the fluoride-mediated toxicity in *Vigna mungo* varieties. For the scope of study two varieties namely *Vigna mungo L.* cultivar KPU 405 (Mukundra urd-2) and PRATAP URD 1(KPU 07-08) were considered. Preliminary pot study was conducted to access fluoride resistance capability of both genotypes. Seeds of both cultivars were sown in soil spiked with varied NaF concentration (0, 10, 20, 30 and 40 ppm). Additionally pots were inoculated with *Bradyrhizobium sp*. in form of a suspension culture, at the 15th day of germination having 160 µmol quanta m^{-2} s^{-1} of photon density, 16-h photoperiod, and 50–60% relative humidity at 28 ± 2 °C. Phytotoxicity of fluoride was observed in terms of oxidative stress among all pots supplied with fluoride while pots with treatment of *Bradyrhizobium sp*. provide reinforcement across all pots among all sets including control groups. The beneficial impact of microbial inoculation was determined based on of different physiological as well as bio-chemical tests including plant biomass, plant height, tolerance index, relative water content, Chl a, chl b and total chl content, decreased the MDA and increased the antioxidant defense system. Interestingly, *Bradyrhizobium sp*. was able to act antagonistically to fluoride stress in both the *Vigna mungo* varieties. The present study concluded that soil application of *Bradyrhizobium sp*. can be a useful approach in protecting *Vigna mungo* from fluoride toxicity.

KEYWORDS: *Bradyrhizobium*, Fluoride, Phytotoxicity, Sustainable, Agriculture

INTRODUCTION

Fluorine is the 13th predominant element in the earth's crust (0.059% by weight) and the 24th prevalent element in the entire cosmos (Singh et al. 2020). Mineral breakdown, industrial waste, and volcanic gas release lead to fluoride building up in the environment over the time. Nearly 300–900 parts per million of fluoride are found in the earth's crust, with the mantle having a 200-fold higher concentration (Johnston and Strobel 2020). Around a quarter of the herbicides used today include fluoride, either as single fluoride atom or as groups like difluoro methyl and trifluoromethyl. Fluoride is harmful to plants, lower crop production as much as half. Plant fluoride concentration increases by 3ppm for every 100ppm increase in soil fluoride, which negatively affects plant health (Alonazi et al. 2025). Salt stress majorly causes phytotoxicity as it negatively affects plant growth and metabolism leading to productivity loss in arid and semi-arid regions (Atta et al. 2023). Salinity induced the generation of reactive oxygen species which results into cytotoxicity (Banerjee and Roychoudhury 2018) as it trends to cause degradation of photosynthetic pigments and lipid peroxidation. Reactive oxygen species cause chlorophyll degradation and membrane lipid peroxidation causing reduced membrane fluidity and selectivity (Hmaeid et al. 2019). Till date, numerous methods have been explored for fluoride bioremediation such as electrodialysis, reverse osmosis, nanofiltration, adsorption, biosorption, ion exchange, etc. (Mukherjee et al. 2016). Despite of the advantageous aspect these conventional approaches possessed certain drawbacks including high financial investment, huge amount of waste generation high water and electricity consumption. Considering these disadvantages, the use of microbes mediated remediation of fluoride is preferred over conventional treatment practices as they trend to bio-transform the salt into lesser toxic byproducts and make it lesser (Chouhan et al. 2012).

Rhizobacteria such as *Bradyrhizobium* aid plant in coping stress by enhancing nutrient uptake by promoting root development (Alharbi et al. 2022; Jarecki 2023). These bacteria are capable of forming symbiotic relationship with plants; increase nitrogen fixation ability and make plant resilient under unfavorable conditions (Lumactud et al. 2023). Additionally, bacterial inoculation

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miraculously boosts soil microbial activity, upscale nutrient cycling and improve plan health (Verma et al. 2022). While nitrogen fixing caliber of *Bradyrhizobium* is well known, recent studies suggest that it may also increase potassium availability and uptake efficiency (Mirriam et al. 2023). However, role of *Bradyrhizobium* in mitigating fluoride stress has been less explored. Thus, this study aims at evaluating the effects of fluoride and *Bradyrhizobium* inoculation on physiological and biochemical attributes of plant growth and development. A greenhouse experiment was conducting using negative controls, fluoride treatments (10, 20, 30 and 40ppm), *Bradyrhizobium* inoculation under saline conditions (10, 20, 30 and 40ppm). Morpho-physiological and certain biochemical response against treatments were records and analysed whether treatment of bacteria can mitigate salt stress and enhance bacterial growth.

MATERIAL AND METHODS

2.1 Sample collection

Seeds of *Vigna mungo L.* varieties KPU 405 (Mukundra urd-2) and Pratap urd 1(KPU 07-08) were precured from agriculture research station, Ummedganj, Kota, Rajasthan, India. The bacterial sample *Bradyrhizobium sp*. (MTCC2380) was collected from Microbial type Culture collection and gene bank, Chandigarh, India.

2.2 Chemicals and Glassware

All the chemicals used in the study were of analytical grade. To prepare the solution, a stock solution of fluoride at 1000 ppm was made by dissolving 2.21 g of anhydrous sodium fluoride in 1000 ml deionized water. Then, different concentrations of fluoride-10, 20, 30 and 40 ppm were made by diluting this stock solution. Culture of *Bradyrhizobium sp*. (MTCC2380) was maintained in Nutrient broth (NB). The laboratory glassware use during the study were washed with detergent and rinse with deionized water followed by oven baking at 200°C overnight, prior to use.

2.3 Plant acclimatization

Seeds of varieties under investigation; *V. Mungo* KPU 405 (Mukundra urd-2) and *V. Mungo* Pratap urd 1(KPU 07-08) were hand-sorted followed by surface sterilization using 0.1% (w/V) of Mercuric chloride solution ($HgCl_2$) for 10 minutes and rinsed with distilled water. After sterilization process, these varieties were preliminary studied for the impact of fluoride in pot experiment. Agricultural soil was air dried and sieved through 4mm sieve. The experiment was planned and carried out in multiple groups (Figure 1). Each pot had 5 kilograms of soil, the seeds were planted in a circle, with 8 to 10 seeds in each pot. Broth culture with 6.7448×10^8cells/ml was added in one set of each variety. Seeds were allowed to germinate at 30±2°C for 6 days under a photoperiod of 14 h. The experiments were terminated on 28th day and the plants were employed for evaluating various physiological and biochemical parameters.

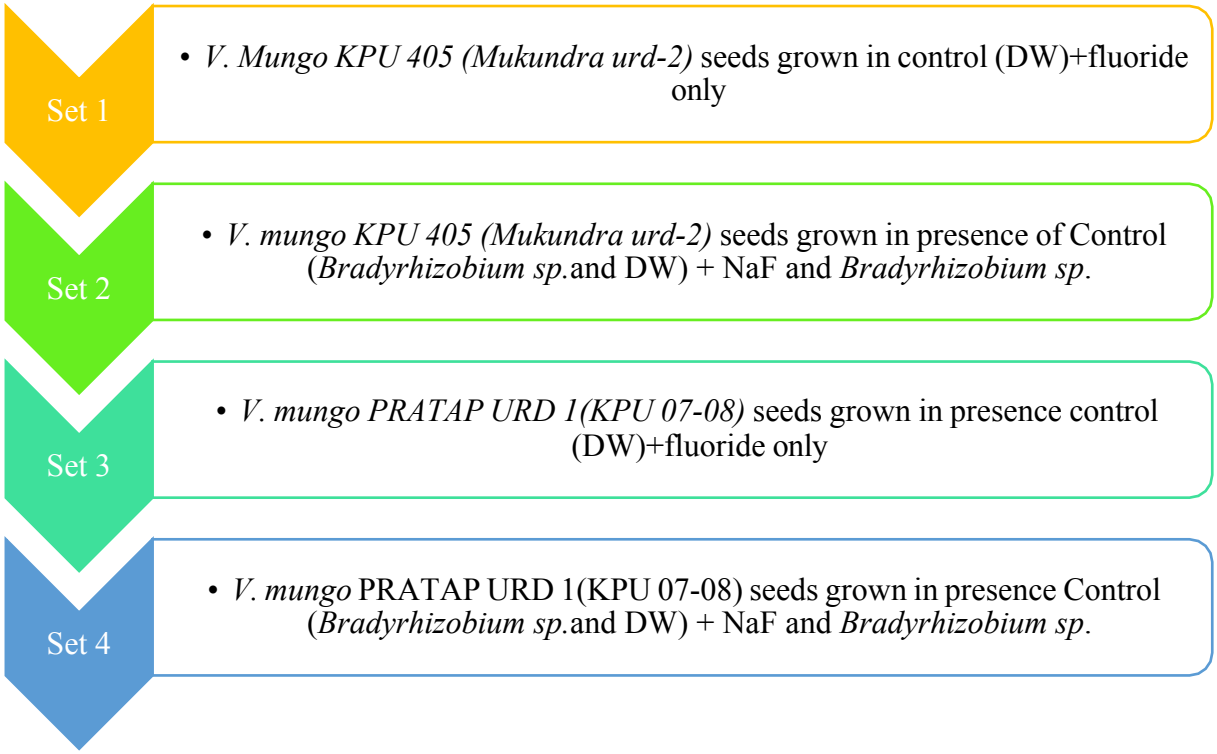

Figure 1: Experimental design

Each set was maintained in triplicate ($n = 3$). The plants were harvested and properly washed under tap water and stored at – 80 °C for further analysis**.** All the plant samples were studied for physiological and biochemical parameters (Figure 2).

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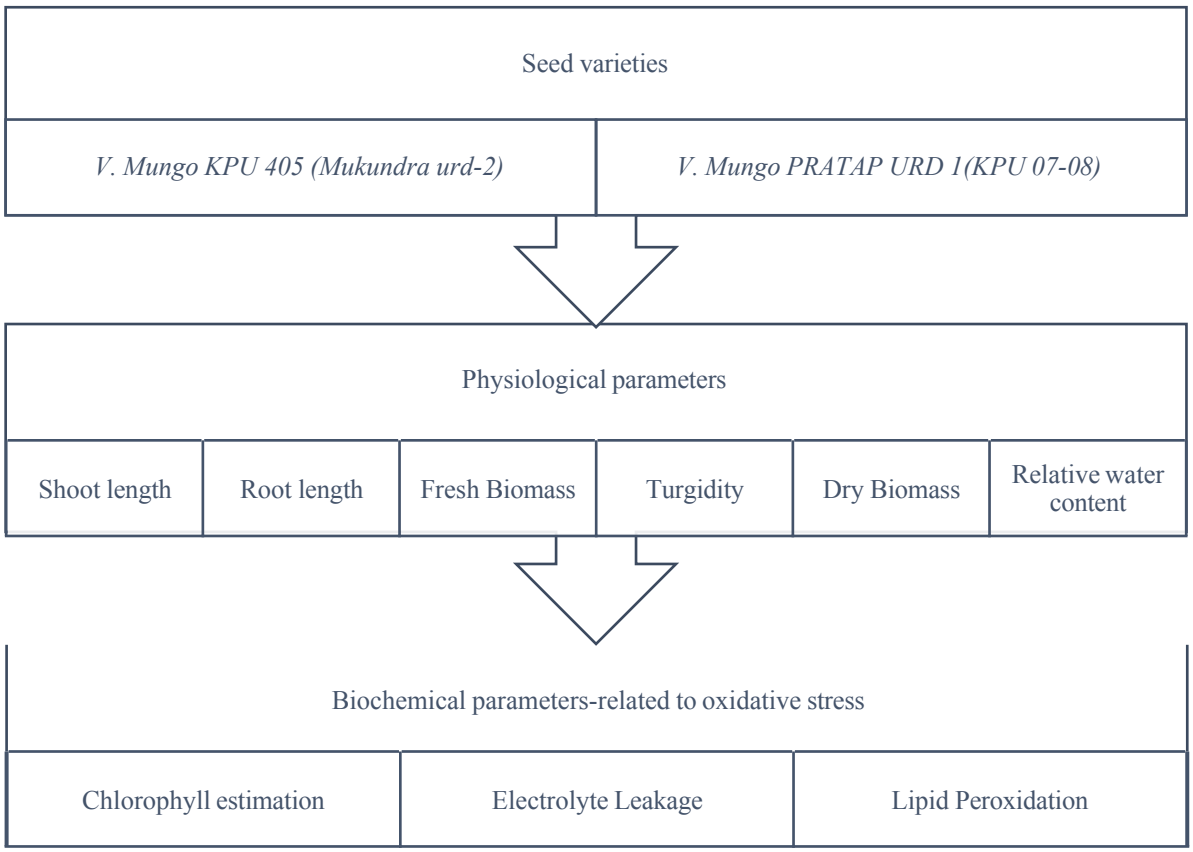

Figure 2: Parameters under study

2.3.1 Physiological parameters

Plant growth and biomass among each treatment were quantified by measuring various physiological parameters including shoot length, root length, fresh weight (Fw), turgid weight (Tw), and dry weight (Dw). Initial stress response in plants was assessed in terms of *plant height stress index* (PHSI) in accordance with the protocol considered by Patel et al. 2024.

PSHI percentage is calculated by multiplying the height of the stressed plant by 100 and then dividing it by the height of the control plant.

Fw was determined immediately after harvest. Tw was recorded after immersing shoots in distilled water for 4 h to ensure full hydration. As for Dw quantification, shoots samples were oven-dried at 80 °C for a period of 48 h until a complete dehydration and then the final Dw of all samples were recorded. The relative water content (RWC) was calculated using the formula (Tahjib-UI-Arif et al. 2018):

$$\textbf{RWC (\%)} = \frac{(Fw - Dw)}{(Tw - Dw)} \times 100$$

2.3.2 Biochemical parameters: related to oxidative stress

2.3.2.1 Chlorophyll Estimation

0.1g of fresh leaves were homogenized using 10ml of 80% acetone in a motor pestle. The homogenized samples were extracted and centrifuged at 10000rpm for 5min. The absorbance of supernatant was recorded at 652, 645 and 663 nm using UV/VIS double bean spectrophotometer (Singh and Roychoudhury 2020). Calculations for Chl a, Chl b and total Chl content was done using mathematical formula given by Arnon, 1949.

$$\text{Chl a} = 12.7 \times (\text{O.D.663}) - 2.69 \times (\text{O.D.645})$$
$$\text{Chl b} = 22.9 \times (\text{O.D.645}) - 4.68 \times (\text{O.D.663})$$
$$\text{Total Chl} = 20.2 \times (\text{O.D.645}) + 8.02 \times (\text{O.D.663})$$

2.3.2.2 Electrolyte leakage (EL)

EL was estimated using the protocol devised by Dionisio-Sese and Tobita (1998) with minor amendments and measured using Electrical conductivity meter asserted in terms of percentage EL. Fresh leaf samples of 100 mg were dissected into small fragments of 5 mm length followed by transfer to test tube containing deionized water. All the test tubes were closed using parafilm and incubated in water bath at 32℃ for 2 h. All the samples were assessed for EL (EC 1) using above method and afterwards, the samples were sterilized at 121℃ for 25 min to eliminate any residual tissue and allowing release all electrolytes. Final EL reading (EL 2) was taken after bringing the samples to room temperature.

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2.3 Lipid peroxidation

Lipid peroxidation can be determined by evaluating the accumulation of reactive 2-thiobarbituric acid (TBA) metabolites, mostly commonly malondialdehyde (MDA) (Heath and Packer 2022). For estimation of lipid peroxidation 0.2g of samples tissue samples were extracted in 0.5 percent of TBA made from 20% TCA and heated at 95°C for 30 min followed by instant cooling. The samples were centrifuged at 10,000 rpm for 10 min. Supernatant was forwarded for spectrophotometry at 532nm and 600nm. The lipid peroxidation was quantified in nmol of MDA when an extinction coefficient of 155 mm^{-1} cm^{-1} is applied (Ansari et al. 2024).

2.4 Statistical analysis

For every observed parameter, mean values and standard deviation were computed from triplicates. The standard deviations (SD) and regression analysis were done in Microsoft Excel 2007. One-way ANOVA analysis was used for the statistical study. Analysis of the data significance was done at 95% confidence levels ($p < 0.05$). Principal component analysis (PCA) and cluster analysis was carried out on nine distinct physiological indicators in order to identify the physiological patterns in a plant. According to Liu et al. (2003), factors loading was categorized as "strong," "moderate," and "weak," which correspond to values of >0.75, 0.75-0.50, and less than 0.50, respectively. Additionally, Pearson's correlation was used to determine how various physiological and biochemical indicators related to one another.

RESULTS AND DISCUSSION

Post exposure of both mung varieties to fluoride stress, both varieties were studied in similar manner to draw a contrast on physiological as well as bio-chemical levels to validate the phytotoxicity the aim of study.

3.1 Physiological Parameters

3.1.1 FW, DW, TW and PHTI

In course of studying the effect of fluoride we found a significant variation ($p<0.05$) level among all tested morpho-physiological parameters. Both the cultivars were observed to exhibit drastic reduction in FW, DW and TW upon exposure of fluoride as compare to controls (Table 1). Previously Chahine et al. 2023 also stated that high Fluoride concentration often leads to declined growth and root-shoot bio-mass (95% and 50% with NaF and KF, respectively) of Jesca. FW of Pratap urd 1(KPU 07-08) decline from 10.62% at 10ppm of fluoride to 36.50% at 40 ppm with an average reduction of 23.91% across all tested concentrations while KPU 405 (Mukundra urd-2) show higher resistance to salt stress as compare to Pratap urd 1 (KPU 07-08) as average reduction in FW was 17.20%. Though both varieties were seen to experience significant toxicity except at 30ppm fluoride concentration where an unexpected raise i.e. 17.13% in FW was observed. This conclusion was suggestive of some adaptive mechanism at this particular concentration. This phenomenon often terms as *hormesis.*

Table 1: Fresh weight, Dry weight and Turgor weight of Pratap urd 1(KPU 07-08) and KPU 405 (Mukundra urd-2)

Pratap urd 1(KPU 07-08)						
	Fresh weight (gm)		**Turgor weight (gm)**		**Dry weight (gm)**	
Conc. (ppm)	**F**	**F+ B**	**F**	**F+ B**	**F**	**F+ B**
C	1.50±0.26	1.80±0.023	1.54±0.305	1.86±0.02	0.237±0.002	0.248±0.059
10	1.34±0.20	1.62±0.034	1.42±0.231	1.63±0.12	0.220±0.03	0.225±0.005
20	1.16±0.16	1.45±0.09	1.29±0.225	1.57±0.11	0.185±0.014	0.257±0.005
30	1.119±0.11	1.31±0.047	1.27±0.08	1.43±0.09	0.184±0.015	0.262±0.020
40	0.956±0.24	0.936±0.090	1.205±0.21	1.08±0.077	0.154±0.03	0.172±0.016
KPU 405 (Mukundra urd-2)						
C	1.28±0.229	2.33± 0.043	1.58±0.202	2.49± 0.09	0.178±0.003	0.357±0.068
10	0.93±0.011	1.574±0.041	1.17±0.037	1.85±0.03	0.158±0.008	0.245±0.041
20	0.883±0.041	1.54± 0.181	1.16±0.037	1.77±0.371	0.167±0.007	0.225±0.006
30	1.39± 0.270	1.41± 0.060	1.54±0.534	1.51±0.212	0.186±0.002	0.204±0.035
40	0.726±0.098	0.93± 0.254	0.98±0.130	1.05±0.380	0.132±0.007	0.174±0.044

Note: Data is presented as mean ±SD from three repeated measurements (n=3), analysed using one way-ANOVA with a p-value of 0.05 or less.

Dry weight measurements confirmed the severe impact of fluoride on biomass accumulation. Pratap urd 1(KPU 07-08) experienced consistent reductions in dry weight across all concentrations, with an average decrease of 21.98%. KPU 405 (Mukundra urd-2) demonstrated better tolerance with an average reduction of 9.70%. Similar trend was observed while studying the shoot length where shoot length decreases drastically across tested fluoride treatments except at 30ppm; average reduction in Pratap urd 1(KPU 07-08) and KPU 405 (Mukundra urd-2) were 20.45% and 9.15%. However, root length among all pot experiment are conclusive of Pratap urd 1(KPU 07-08) experiencing 18.75% and KPU 405 (Mukundra urd-2) 21.54% percentage reduction (Table 2). Thus, there is a need to explore sustainable, greener and innovative methods to protect plant from toxicity caused by salt stress.

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Table 2: Shoot length, PHTI and root length of Pratap urd 1(KPU 07-08) and KPU 405 (Mukundra urd-2)

Pratap urd 1(KPU 07-08)	Shoot length (cm)		PHTI (%)		Root length (cm)	
Conc. (ppm)	F	F+ B	F	F+ B	F	F+ B
C	20.13±0.46	19.56±0.89	ND	ND	12.53±3.05	14.7±0.472
10	17.63±1.15	15.5±1.00	89.65±0.63	87.94±0.31	10.33±0.05	8.9±0.519
20	16.8±0.83	15.1±0.529	89.10±0.08	77.82±1.09	7.4±0.92	7.7±0.404
30	16.26±2.89	16.13±0.75	96.29±0.05	89.93±1.00	10.8±0.173	12.4±1.154
40	13.4±1.96	13.83±0.35	65.07±1.12	71.54±1.76	10.9±1.84	11.26±3.34
KPU 405 (Mukundra urd-2)						
C	18.04±0.36	21.1±1.25	ND	ND	12.26±0.60	8.8±0.346
10	16.3±0.173	19.14±1.71	81.88±2.93	91.88±2.10	11.56±0.46	9.0±0.404
20	15.4±0.624	16.4±1.41	79.33±1.44	89.56±1.89	8.2±2.59	10.5±3.5
30	21.7±0.288	20.86±0.70	116.27±2.05	91.31±0.39	7.46±2.02	6.46±0.20
40	13.4±0.152	13.3±0.85	69.15±1.64	75.81±0.20	9.26±1.32	10.0±1.7

Note: Data is presented as mean ±SD from three repeated measurements (n=3), analysed using one way-ANOVA with a p-value of 0.05 or less.

The results when contrasted with experimental group of only fluoride stress provide enough evidence that treatment of bacteria significantly reduces the toxic effect of fluoride. Our findings were in line with previous study conducted by Singh et al. 2024 on phytotoxicity of fluoride on *Lycopersicon esculentum* Mill. and ameliorative role of *P. aeruginosa* strain. Apart from these various plants have been studied previously to study phytotoxic effects of stress inclusive *Sulla carnosa* (Hmaeid et al. 2019), wheat (*Triticum aestivum*) (Ansari et al. 2019) and rice (*Oryza sativa*) (Sarkar et al. 2018). Our approach of employing bacterial isolate for mitigating the toxicity caused by fluoride was supported by Zulfiqar et al. (2023) who utilized fluoride-resistant bacterial strains (SS-10a and SS-5a) in the soil.

Generally, plant growth promoting rhizobacteria (PGPR) have innate mechanism, to cope with stressed conditions. Under fluoride stress conditions PGPR utilize their channel proteins, putative F- transporters which often pump out excess salt from bacterial cell (Chellaiah et al. 2021). Recent research suggests that certain microbes have evolved a unique mechanism to effectively transport fluoride, which enables them to thrive in fluoride-rich environments. Moreover, these microbes seem to possess the ability to identify specific targets for fluoride, which could have significant implications for comprehending their survival strategies in such harsh conditions. PHTI in KPU 405 (Mukundra urd-2) cultivar was higher than that of Pratap urd 1(KPU 07-08) after microbial inoculum which suggest positive correlation between bacterial population and plant's ability to fight against fluoride stress. Above results were indicative of higher stress experienced by Pratap urd 1(KPU 07-08) and KPU 405 (Mukundra urd-2) being more resistant owing to better stress coping responses (Figure 1).

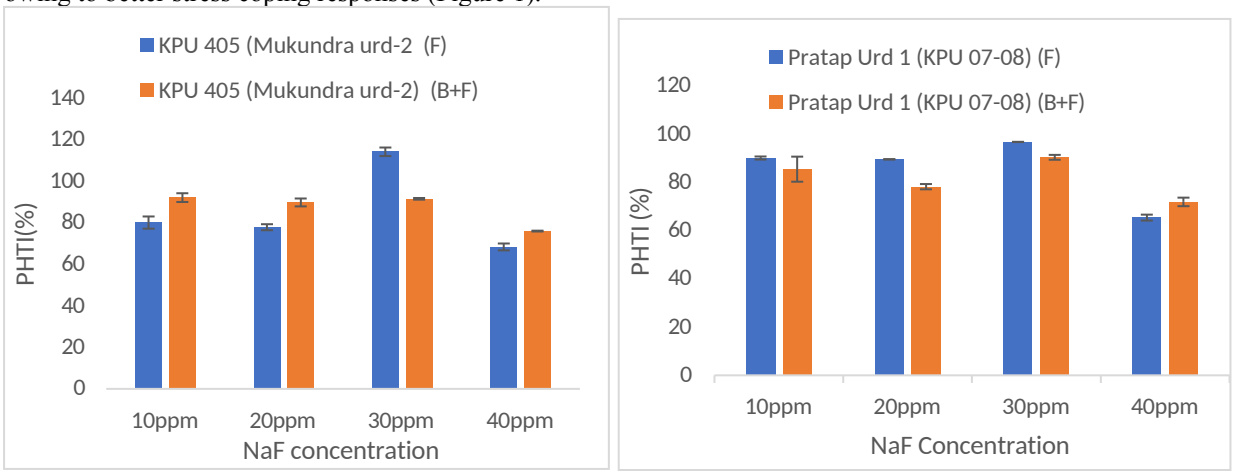

Figure 1. Effect of *Bradyrhizobium* sp. on PHTI of KPU 405 (Mukundra urd-2) and Pratap urd 1(KPU 07-08)

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3.1.2 Water relation Impairment

RWC provide status of water in plant under salt saturation conditions. RWC analysis unraveled the negative aftermath of fluoride treatment on both cultivars. Plant of KPU 405 (Mukundra urd-2) with treatment of NaF and *Bradyrhizobium* were observed to maintain higher RWC as compared to plants pointing towards positive influence of treatment on tendency of water uptake and retention (Figure 2).

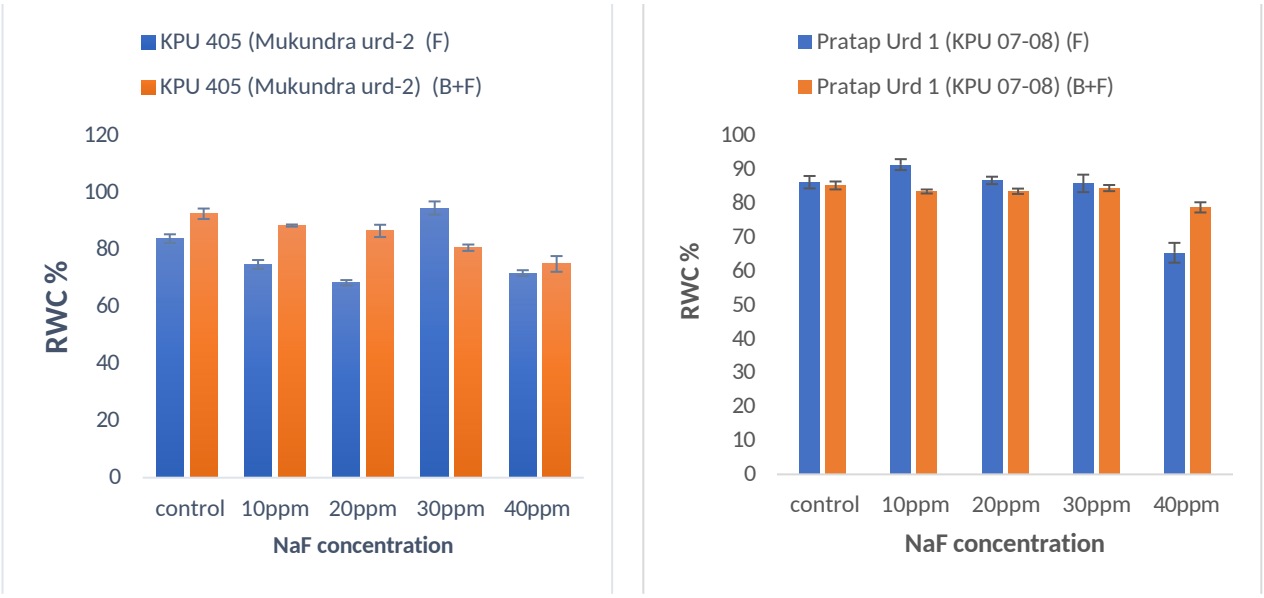

Figure 2: Effect of *Bradyrhizobium sp*. on Relative water content of KPU 405 (Mukundra urd-2) and Pratap urd 1(KPU 07-08)

However, the findings of Pratap urd 1(KPU 07-08) were not very conclusive (Table 3). Higher RWC plays a significant role in promoting plant growth and development. It can be reasoned with the enhanced rate of transpiration or increased water efficacy. Both the conditions are known to substantially contributes to overall stress tolerance bestowed by *Bradyrhizobium*.

Table 3: MDA content, Electrolyte leakage and Relative water content of Pratap urd 1(KPU 07-08) and KPU 405 (Mukundra urd-2)

Pratap urd 1(KPU 07-08)						
	Chlorophyll a		**Chlorophyll b**		**Total chlorophyll**	
Conc. (ppm)	**F**	**F+ B**	**F**	**F+ B**	**F**	**F+ B**
C	20.11±1.05	26.47±0.24	8.94±1.87	10.38±2.18	32.17±1.01	39.20±0.010
10	18.96±0.26	21.33±0.52	6.82±1.11	7.78±0.10	26.36±0.14	28.85±0.005
20	15.41±2.06	23.28±1.15	6.01±0.61	7.75±0.09	24.23±0.11	30.96±1.74
30	15.77±0.36	21.56±0.90	6.19±1.74	6.20±0.09	24.12±1.73	29.13±1.87
40	18.54±0.05	20.65±2.50	6.39±1.05	6.98±0.008	23.75±2.25	27.77±2.04
KPU 405 (Mukundra urd-2)						
C	17.20±0.60	20.93±1.83	7.03±0.61	7.17±0.28	24.74±1.28	28.60±1.19
10	15.06±0.71	18.39±1.04	6.18±0.57	7.23±0.41	22.85±0.55	24.28±0.33
20	14.77±1.79	16.15±0.46	5.59±1.19	6.47±0.54	20.79±0.25	24.99±0.38
30	21.95±1.84	19.04±0.75	8.41±1.69	9.03±0.28	30.82±2.94	29.33±3.52
40	12.70±0.08	20.06±0.70	5.93±0.39	6.90±0.66	16.71±2.49	19.95±0.68
Note: Data is presented as mean ±SD from three repeated measurements (n=3), analysed using one way-ANOVA with a p-value of 0.05 or less.						

3.2 Damage assessing parameters: Chlorophyll, Electrolytic leakage and MDA

3.2.1 Chlorophyll content

The concentration of photosynthetic pigment was greatly influenced by the fluoride (Table 3). The data analysis clearly shows that under NaF treatment group, there was a significant drop in Chl a, Chl b and total Chl content in contrast with control pointing towards inverse relationship between photosynthetic pigment and fluoride concentration. The results were found to fall in line with previous

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research by Saleem et al. 2021 on *Gossypium hirsutum,* Singh et al. 2024 on Tomato, Turan et al. (2007) on *P. vulgaris* L. and Taffouo et al. (2010) on *Vigna subterranean* L. which clearly mentioned the oxidative stress caused by salt stress. Our remedial approach of inoculating both legume varieties with *Bradyrhizobium*, remarkable improve the concentration of photosynthetic pigment across all tested concentration as depicted in Table 3 and Figure 3. Chlorophyll content analysis revealed interesting cultivar-specific patterns. For chlorophyll a, Pratap urd 1(KPU 07-08) surprisingly outperformed KPU 405 (Mukundra urd-2) with 27.58% average increase compared to 15.78%. However, for total chlorophyll content, KPU 405 (Mukundra urd-2) maintained superiority (9.70% vs 8.13%)

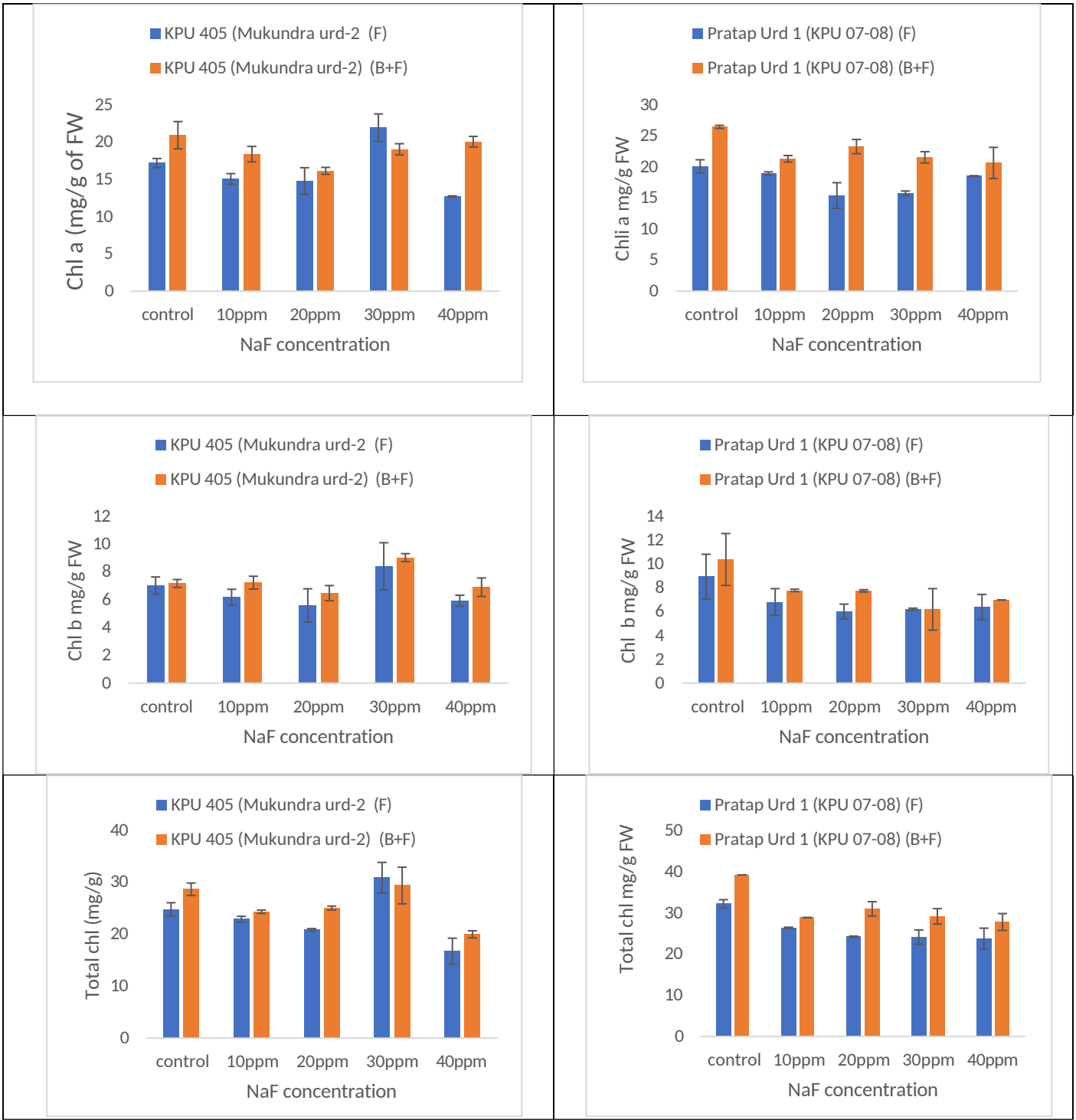

Figure 3. Effect of *Bradyrhizobium* sp. on (a) Chl a, (b) Chl b, (c) Total Chl of KPU 405 (Mukundra urd-2) and Pratap urd 1 (KPU 07-08) under fluoride stress. Data is shown as Mean ± S.D of triplicate values (n=3), One-way Anova, p-value ≤0.05

3.2.2 Electrolyte Leakage (EL) and MDA

Electrolyte leakage (EL) is key parameter aiding in assessment of injury to plasma membrane. Scrutinizing EL test has been regarded as a quantifiable, genuine, scalable and simple test for evaluating cell viability after thermal (Heat or cold stress) and salt (Jamal et al. 2014; Ullah et al. 2014). Plasma membrane readily denatures or aggregates under unfavorable condition; stress owing to its phospho-lipid components leading to *hyperfluidity* of lipid components. (Guo et al. 2019). As expected, both the cultivars were

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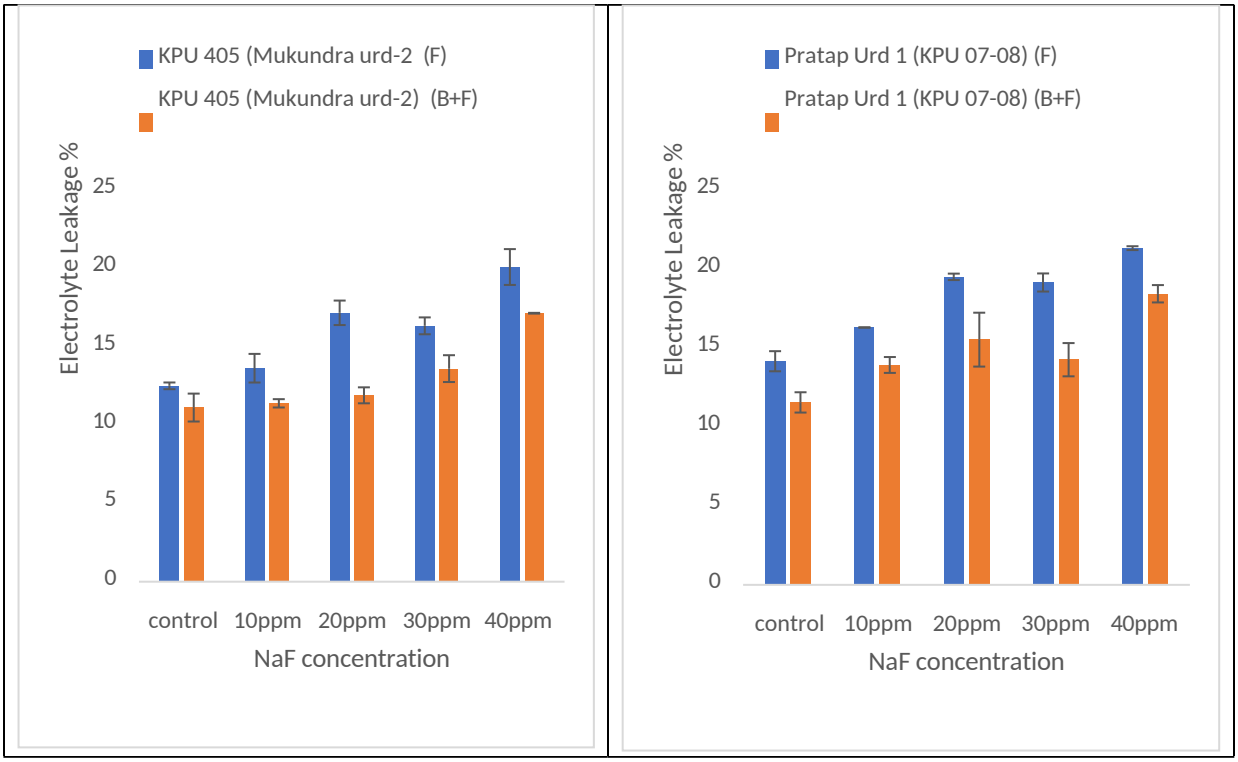
seen to follow the trend observed in prior research as cell permeability and cellular damage were the resultants of fluoride stress. However, post inoculation of *Bradyrhizobium* there was a notable decrease in EL as compared to the control (Table 4, Figure 4). Our findings were in sync with Alexander et al. 2020 and Saleem et al. 2021.

Table:4 MDA content, Electrolyte leakage and Relative water content (RWC) of Pratap urd 1(KPU 07-08) and KPU 405 (Mukundra urd-2)

Pratap urd 1(KPU 07-08)						
	MDA content (µM/g)		**Electrolyte leakage (%)**		**RWC (%)**	
Conc. (ppm)	**F**	**F+ B**	**F**	**F+ B**	**F**	**F+ B**
C	0.58±0.08	0.18±0.002	14.13±0.63	11.53±0.64	81.15±1.82	85.19±1.17
10	0.55±0.05	0.27±0.05	16.26±0.01	13.89±0.50	91.4±1.60	83.40±0.59
20	0.59±0.08	0.48±0.05	19.46±0.20	15.49±1.70	86.6±1.09	83.47±0.78
30	0.89±0.05	0.79±0.18	19.11±0.57	14.23±1.05	85.8±2.57	84.41±0.90
40	0.445±0.01	0.50±0.021	21.28±0.12	18.39±0.54	65.4±2.97	78.72±1.50
KPU 405 (Mukundra urd-2)						
C	0.20±0.02	0.12±0.05	12.47±0.21	11.09±0.88	83.79±1.50	92.5±1.82
10	0.48±0.11	0.32±0.08	13.6±0.91	11.36±0.26	74.74±1.53	88.36±0.36
20	0.59±0.07	0.30±0.07	17.13±0.77	11.87±0.50	68.30±0.94	86.48±2.15
30	0.24±0.02	0.26±0.04	16.3±0.53	13.57±0.85	94.5±2.32	80.6±1.11
40	0.57±0.01	0.34±0.019	20.04±1.14	17.10±0.02	71.72±0.988	74.91±2.75

Note: Data is presented as mean ±SD from three repeated measurements (n=3), analysed using one way-ANOVA with a p-value of 0.05 or less.

Lipid oxidase is most ascribed symptom of oxidative damage thus, has been profoundly employed to assess the oxidative stress in studying plant stress physiology. Malondialdehyde (MDA) content analysis revealed interesting cultivar-specific responses to fluoride-induced oxidative stress. Pratap urd 1(KPU 07-08) observes to show lower stress response as 5.75% average increase in MDA with 50% of parameters negatively affected, while KPU 405 (Mukundra urd-2) exhibited a dramatic 126.47% average increase with 100% of measurements showing elevated oxidative stress markers.



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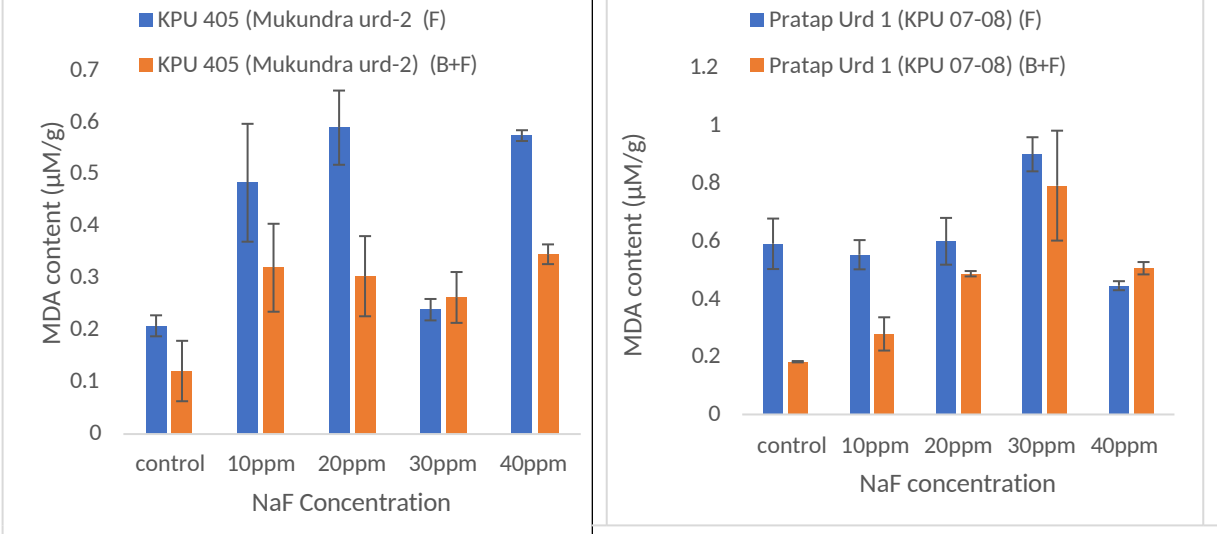

Figure 4: Effect of *Bradyrhizobium* in mitigating fluoride toxicity (a) Electrolyte leakage (b) MDA content of KPU 405 (Mukundra urd-2) and Pratap urd1 (07-08). Data is shown as Mean ± S.D of triplicate values (n=3), One-way Anova, p-value ≤0.05.

The addition of bacterial inoculum dramatically reduces the MDA levels as compare to respective controls suggesting better antioxidant defense activation. bioaccumulation of fluoride known to trigger oxidative stress in plant tissues which in turn accelerates the formation of reactive Oxygen species (ROS) such as hydrogen peroxide (H_2O_2). High ROS activates lipoxygenase responsible for peroxidation of lipid components of cell membrane thereby cause enhanced levels of malondialdehyde (MDA) and results in membrane disruption (Banerjee and Roychoudhury, 2019). Additionally, oxidative stress leads to accumulation of certain cytotoxic compounds inclusive of methylglyoxal (MG) (Banerjee and Roychoudhury, 2018) and act as inducer for carbonylation reaction of certain amino acid in proteins which leads to disintegration of structure of protein; enhanced susceptibility to protease activity (EC.3.4.21).

3.3 Statistical analysis

3.3.1 Concentration-Dependent Effects

The dose-response analysis revealed that 20ppm fluoride was the most toxic concentration overall, affecting 95.5% of measured parameters. The 10ppm and 40ppm concentrations showed 90.9% and 95.5% toxicity rates respectively, while 30ppm demonstrated the lowest toxicity at 63.6%. This non-linear dose-response relationship suggests complex physiological interactions at different fluoride concentrations.

3.3.2 Photosynthetic Function

Photosynthetic parameters were severely compromised, with Pratap urd 1(KPU 07-08) showing 100% of parameters negatively affected and 23.86% average reduction, compared to KPU 404 (Mukundra urd-2)'s 75% affected parameters and 7.07% average reduction. This indicates that fluoride significantly disrupts chlorophyll biosynthesis, chloroplast structure, and photosynthetic electron transport chains.

3.3.3 Effect of *Bradyrhizobium sp.* in ameliorating fluoride toxicity

KPU 405 (Mukundra urd-2) demonstrated superior responsiveness with 75% of parameters showing improved performance compared to only 25% in Pratap urd 1(KPU 07-08). The overall success rates were dramatically different, with KPU 405 (Mukundra urd-2) achieving 91.7% beneficial effects across all measured parameters, while Pratap urd 1(KPU 07-08) showed only 58.3% beneficial effects.

The effectiveness of *Bradyrhizobium* treatment varied significantly with fluoride concentration and cultivar. KPU 405 (Mukundra urd-2) showed optimal responses at 20ppm and 40ppm concentrations (100% success rate), while exhibiting reduced effectiveness at 30ppm (33.3% success rate). Pratap urd 1(KPU 07-08) maintained more consistent but lower success rates across concentrations (58.3-66.7%), with the best performance at 30ppm concentration.

3.4 Statistical analysis

3.4.1 Pearson correlation matrix

The correlation analysis revealed two distinct biological patterns: biomass variables (fresh weight, total weight, dry weight) and shoot length showed very strong positive correlations ($r > 0.86$ to 0.88), indicating coordinated growth processes, while physiological health indicators (PHSI, RWC) and chlorophyll content correlated moderately with growth parameters (r 0.48-0.85) (Figure 5). Highlighting that higher water retention and healthier physiological status promote growth.

In contrast, stress indicators displayed inverse relationships—electrical conductivity strongly negatively correlated with growth and chlorophyll (r -0.70 to -0.77), while MDA showed moderate negative correlations with biomass. Notably, root length exhibited weak correlations with most parameters, suggesting independent root regulation from overall plant growth. This pattern demonstrates that plant growth components move together under favourable conditions, while stress disrupts this coordination and triggers independent physiological responses.

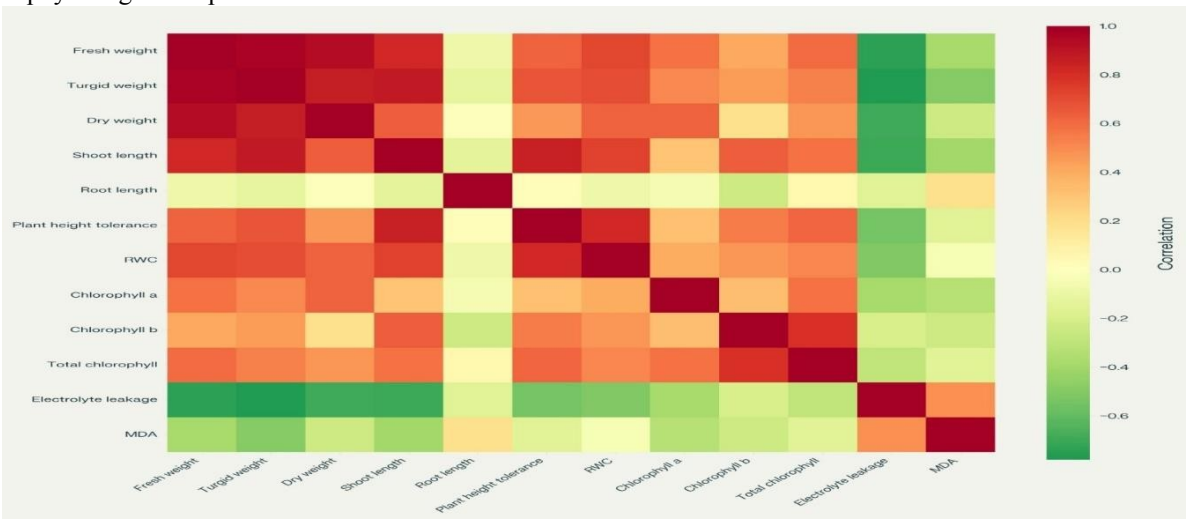

Figure 5: Pearson's correlation matrix of the physiological parameters studied across different treatments in *Vigna mungo* genotypes KPU 405 (Mukundra urd-2) and Pratap urd 1 (KPU 07-08)

3.4.2 Principal component analysis and cluster analysis

The PCA shows that the first two components capture most variance (PC1: 55.98%, PC2: 11.00%; cumulative 66.98%), rising to 77.13% with PC3. PC1 reflects overall plant health and growth under fluoride stress, while PC2 represents biochemical stress responses. Genotypes treated with *Bradyrhizobium* + fluoride (Pratap urd 1(KPU 07-08) B+F: 0.52; KPU 405 (Mukundra urd-2) B+F: 1.50) cluster toward tolerance, whereas fluoride alone (KPU 405 (Mukundra urd-2) F: –1.41; Pratap urd 1(KPU 07-08) F: –0.61) indicates sensitivity. *Bradyrhizobium* inoculation markedly mitigates fluoride toxicity (Figure 6).

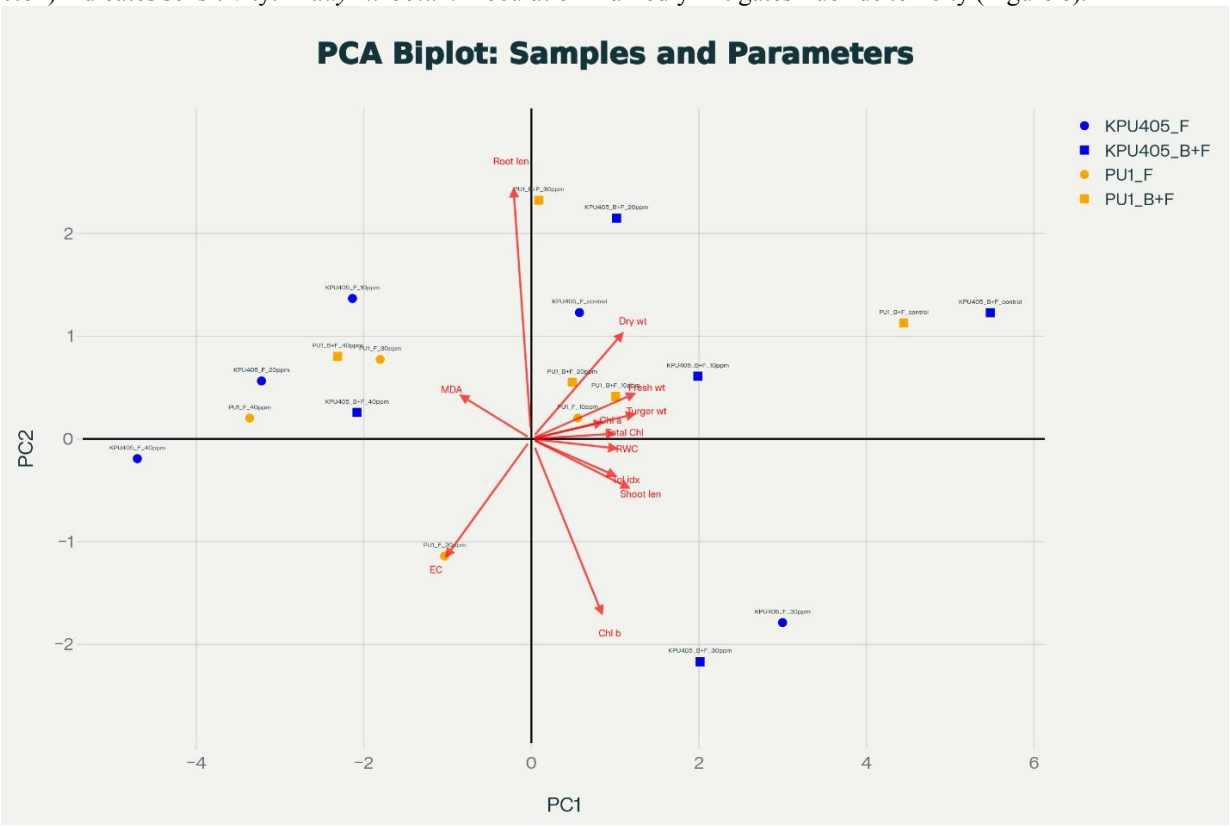

Figure 6: The main PCA biplot shows the separation of treatments in the PC1-PC2 space, with loading vectors indicating how each physiological parameter contributes to the separation. This visualization clearly demonstrates the clustering of treatments by stress level and genotype

Hierarchical and K-means clustering both achieved good quality (silhouette ≈0.51) and strong-to-moderate agreement with

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known groups (ARI 0.693 vs. 0.559). Samples cluster by genotype–treatment, with *Bradyrhizobium* sp. + fluoride (B+F) forming distinct groups from fluoride-only, high fluoride doses (40 ppm) grouping together, and clear separation between KPU 405 (Mukundra urd-2) and Pratap urd 1(KPU 07-08) (Figure 7). This confirms *Bradyrhizobium* sp. protective effect against fluoride stress.

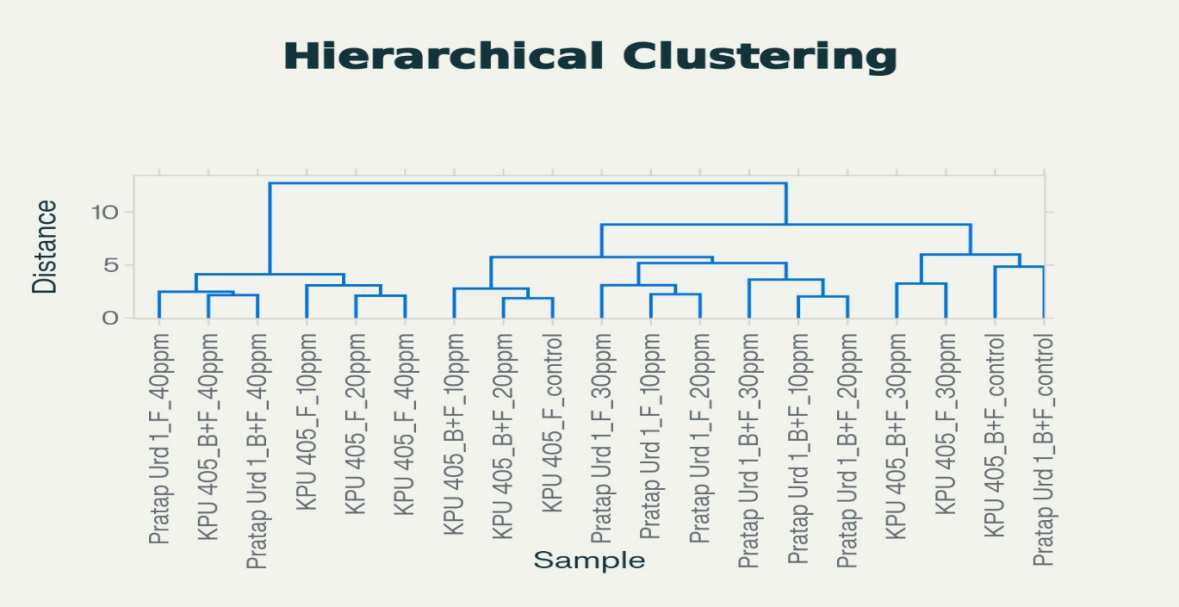

Figure 7: Hierarchical Dendrogram Illustrating Clustering of Pratap urd 1(KPU 07-08) and KPU 405 (Mukundra urd-2) Genotypes under *Bradyrhizobium sp.* (B) and Fluoride (F) Treatment Combinations

3.4.3 Regression analysis

The linear regression model explained 91.7% of variance ($R^2 = 0.917$), with shoot length ($p = 0.058$) and RWC ($p = 0.083$) as the strongest predictors.

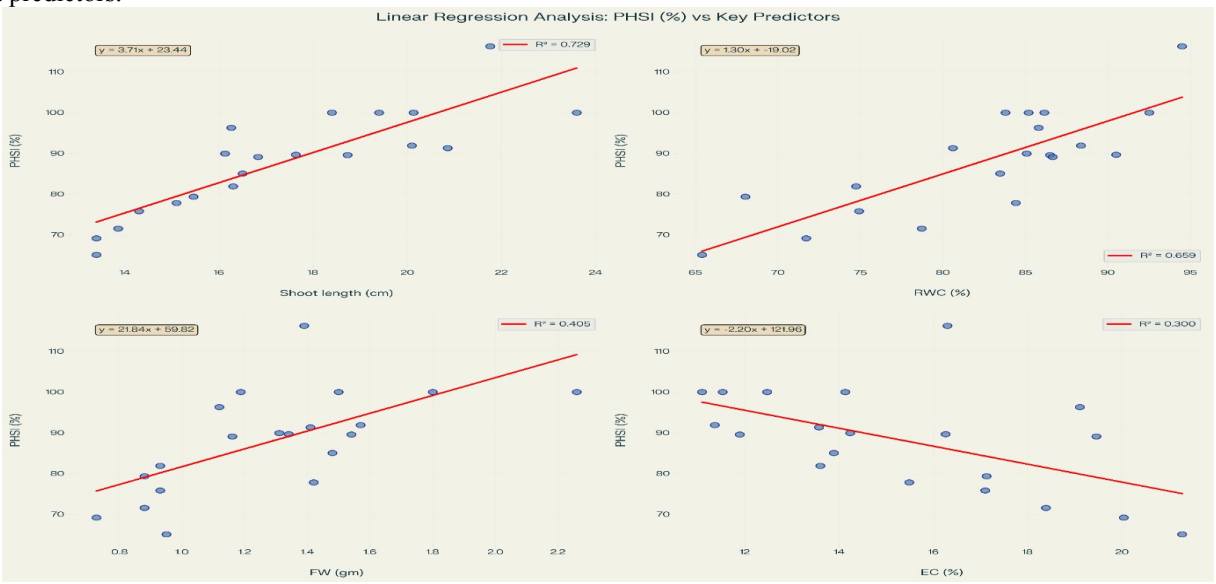

Figure 8. Linear Regression of Plant Health Stress Index (PHSI) Against Key Predictors This four-panel plot shows how PHSI (%) relates to four key predictors: (a) Shoot Length (R^2=0.729): Taller plants have higher stress-tolerance scores (b) RWC (R^2=0.659): Better water status strongly supports plant health (c) Fresh Weight (R^2=0.405): More biomass moderately predicts higher PHSI (d) EC (R^2=0.300): Increased electrolyte leakage weakly indicates lower plant health.

Residuals were normally distributed with no heteroscedasticity, confirming that morphological and physiological traits reliably predict fluoride stress tolerance (Figure 8).

CONCLUSION

This study firmly supports our hypothesis that introduction of *Bradyrhizobium* aid in coping fluoride stress. Statistical analysis was evident of cultivar-specific responses to *Bradyrhizobium* inoculation, as KPU 405 (Mukundra urd-2) exhibits higher overall performance enhancement such as plant growth, enhanced water relations, accelerated photosynthetic capacity and enhanced tolerance against salt stress in contrast to Pratap urd 1(KPU 07-08). The results were conclusive of significance of considering plant

genotype as important aspect in biological inoculation programs specifically those based on stress biology. As the synchrony of bacterial inoculation and plant genetics are key factors effecting the results of such programs. Furthermore, the real time studies may show less effectivity as the standardization of bacterial activity for mitigation of fluoride *in-vivo* is often tedious procedure. The comprehensive improvements observed in KPU 405 (Mukundra urd-2), including enhanced growth, improved water relations, increased photosynthetic capacity, and better stress tolerance, demonstrate the potential for developing highly responsive cultivars that can maximize the benefits of plant growth-promoting bacteria while reducing dependence on chemical inputs.

DECLARATIONS

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Author contributions

Aziz Mohammed Khan conceived and designed the whole study. Parul Gautam conducted the study, wrote the first draft. Data validation and analysis was done by Akriti Singh and Sushma Gautam. Final proof reading was conducted by Aziz Mohammed Khan.

Conflict of Interest

Author's share none competing or conflicting interest

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