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Influence of Water Stress and Rhizobial Inoculation on Accumulation of Proline in Selected Cultivars of *Phaseolus vulgaris* (L.)

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ABSTRACT

Keywords

Drought, Common bean, Inoculants, Varieties, Water.

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A two season field experiment and a single season screen house experiment were conducted to assess the effect of water stress periods and rhizobial inoculation in five (5) *P. vulgaris* (L.) cultivars. The experiment consisted of 2 levels of rhizobia (with and without inoculation), two stress levels (With and without stress) and five cultivars of *P. vulgaris* (L.) (*KAT B9*, *KAT B1*, *F9 Kidney Selection*, *F8 Drought Line* and *JESCA*). The field experiment was conducted for two consecutive seasons, while the screen house study was done in a season. Results showed that proline content ($\mu\text{mol g}^{-1}\text{.FW}$) was higher in inoculated and water stressed treatments. Variety number 4 (*F8 Drought Line*) and 5 (*JESCA*) significantly recorded higher proline content in field experiment as compared to the rest. However, in the screen house experiment, variety 2 (*KAT B1*) and 4 (*F8 Drought Line*) significantly accumulated more proline than the other tested varieties. Significant interactive effects were also observed between inoculation, water stress periods and the tested *P. vulgaris* varieties.

Introduction

Proline is an organic osmolyte, N containing compound which stand as osmoprotection agent involved in reducing oxidative damage in plants by reducing free radicals (Tatar and Gevrek, 2008; Matysik *et al.*, 2002). Apart from acting as an osmolyte, proline accumulation has other important cell functions. Proline tends to act as N source in the cell under stress conditions, where the accumulation of this nitrogenous compound could be utilized as a form of stored N (Dandekar and Uratsu, 1988). Under condition of N deficit, proline accumulation in plant declines which implies that the

degradation of proline is influenced by the stimulation of the enzyme proline dehydrogenase. However, under condition of sufficient N, proline level increase due to the action of ornithine, signifying majority of the ornithine pathway over the glutamine pathway, in addition to the inhibition of proline dehydrogenase activity (Sánchez, *et al.*, 2002). Elboutahiri *et al.*, (2010) reported that *Rhizobium* inoculated alfalfa had the highest leaf proline levels. Generally, N deficiency is characterized by a decrease in proline accumulation in plant tissues, essentially

because the degradation of proline is favoured by the stimulation of proline dehydrogenase. Proline in plant is synthesized mainly from glutamate (pyrroline-5-carboxylate (P5C), synthetase (P5CS) and P5C reductase (P5CR) and converted back into glutamate by proline dehydrogenase (PDH) and P5C dehydrogenase (Szabados and Savoure, 2009; Delauney and Verma, 1993; Kishor *et al.*, 2008). From the above background, inoculating legumes with appropriate rhizobial strain may result in more accumulation of proline in plant tissues and hence rendering them tolerant to water stress.

Abiotic stress condition such as water limitation in higher plants result in huge accumulation of plant osmolytes mainly proline and glycine betaine (Kavikishor *et al.*, 2005). Majority of plants accumulate compatible osmolytes like proline (Pro), Glycine betaine and sugar alcohols, when they are exposed to water stress and/or drought (Tatar and Gevrek, 2008). Proline among other amino acids is commonly produced in higher plants and generally accumulates in large extent in response to environmental stresses (Ashraf and Foolad, 2007). Proline play a very important role in plants, a part of osmolyte for osmotic adjustment, it stabilize sub cellular structures such as membrane and proteins and scavenging free radicals (Matysik *et al.*, 2002; Tatar and Gevrek, 2008; Mafakheri *et al.*, 2010). It also contribute in alleviating cytoplasmic acidosis and maintaining appropriate $NADP^+/NADPH$ ratios compatible with metabolism (Hare and Cress, 1997). According to Stewart (1981), proline does not hamper with normal biochemical reactions but allows the plants to survive under stress. Studies have reveal that proline perform as solute during stress, where an increase in the proline content would indicate resistance or tolerance to water deficit, serve as parameter for the assortment of highly

resistant cultivars (Bates *et al.*, 1973). For example, the proline content increased under drought stress in pea (Sanchez *et al.*, 1998). In higher plants, accumulated proline can have many other important functions, prevention of membrane disintegrations and enzyme inactivation in the environment of low water activity.

Once plants accumulate proline in their tissues, the proline tends to reduce the toxic effects of ions in enzymes activity and also lowers the generation of free radicals formed by abiotic stresses (Siddiqui *et al.*, 2015). The theory behind proline is therefore very useful to assess the physiological status and more generally to understand stress tolerance in plants species. Therefore the aim of this work is to assess the effects of water stress/drought among the five (5) common bean varieties as influenced by stress phases and rhizobial inoculation respectively.

Materials and Methods

Description of site location

The trial was conducted at Agricultural Seed Agency (ASA) farm in Arusha, located at Latitude 3°18'S and Longitude 36°38'06.29"E. ASA receives the mean annual rainfall of 819mm, mean temperature of 19.15°C with relative humidity of about 94% and altitude of 1520 m.a.s.l. The field trial was carried out during dry season of January, to March 2014 and January, to March, 2015 while the screen house experiment was carried out from mid January to March, 2016 under irrigation.

Experimental design and treatment application

The experiment was designed in split, split plot with 3 replications. The plot size was 3 x 4m. The field experimental treatments

consisted of 2 levels of Rhizobia (with and without inoculation) as the main factor followed by imposing of stress (sub factor) in vegetative and flowering stages of plant growth. Five cultivars of *P. vulgaris* (L.) (*KAT B9*, *KAT B1*, *F9 Kidney Selection*, *F8 Drought Line* and *JESCA*) were assigned to sub-sub plots. The common bean seeds were sown at a spacing of 50 cm x 20 cm, making a plant population density of 200,000 plants per hectare. The BIOFIX legume inoculants were obtained from MEA Company Nairobi-Kenya, sold under license from the University of Nairobi. Common bean seeds lines and/or varieties *KAT B9*, *KAT B1*, *F9 Kidney Selection*, *F8 Drought Line* and *JESCA* were obtained from the breeding unit based at Selian Agricultural Research Institute (SARI), Arusha, Tanzania.

Land for field experiment was cleared and all the necessary practices like ploughing and harrowing were done before planting. Moreover, in the screen house experiment, wooden box technique was used to establish the experiment. This was done by collecting the same soil used at field experiment and beans were planted using the protocol developed by Agbicodo *et al.*, (2009) with some modifications. Common bean seeds were thoroughly mixed with *R. leguminosarum* inoculants to supply (10^9 cells/g seed), following procedure stipulated by products manufacturer. To avoid contamination, all non-inoculated seeds were sown first, followed by inoculated seeds. Three seeds were sown and thinned to two plants per hill after full plant establishment. Stress period of 10 days were imposed at vegetative and flowering stages of plant growth by not irrigating.

Plant harvest and sample preparation

Plant leaf samples from field and glasshouse experiments were collected for proline

analysis. In the field experiment, 10 plants were randomly sampled from the middle rows of each plot while in the glasshouse experiment two plants from each pot were sampled. The fresh plant leaf samples from each of the growth stages (i.e. vegetative and flowering) were collected from the third young leaf from the top and kept in ice container to maintain their freshness for proline determination.

Determination of proline contents in plant leaves

Extraction of proline contents in plant leaves was done as described by Bates *et al.*, (1973). Extract of 0.5g of plant material were homogenized in 10mL of 3% aqueous sulphosalicylic acid. The homogenate were filtered through Whatman No. 2 filter paper. The 2mL of filtrate were taken in a test tube and 2mL of glacial acetic acid were added followed by 2mL acid ninhydrin. The mixture was then heated in the boiling water bath for 1 hour. The reaction was then terminated by placing the tube in ice bath and 4mL of toluene was added to the reaction mixture and stirred well for 20-30 seconds. The toluene layer was separated and warmed to room temperature. The red color intensity was then measured at 520 nm using 2800 UV/Vis Spectrophotometer. Standard curve were then prepared and the amount of proline in the test sample were obtained from the standard curve. The proline content on fresh-weight basis was calculated as follows; $\mu\text{moles/gram tissues} = [(\mu\text{gproline/ml}) \times \text{ml toluene}] / [115.5\mu\text{g}/\mu\text{mole}] / [(\text{g. sample})/5]$

Statistical analysis

A 3-way ANOVA was used to analyze data collected. The analysis was done using STATISTICA software program of 2013. Fisher's least significant difference was used to compare treatment means at $p = 0.05$ (Steel and Torrie, 1980).

Results and Discussion

Effect of inoculation with *R.leguminosarum biovar phaseoli* and stress periods on proline content in selected *P. vulgaris* (L.) varieties

Significance increase in proline content ($\mu\text{mol g}^{-1}\text{FW}$) was observed in inoculated compared with non-inoculated treatments (Table 1 & 2). Rhizobial inoculation significantly increased proline content during vegetative stage by 12% and 8% in season one and two respectively (Table 1). In screen house

experiment, inoculation with *Rhizobium* strain increased the proline content by 34% in vegetative stage and 31% in flowering stage when compared with un inoculated treatments (Table 2). Water stress treatments significantly increased proline content by 35 and 39% in season one and by 33 and 48% in season two at vegetative and flowering stages respectively (Table 1). In the screen house experiment, water stress treatment increased the proline levels in plants by 36% and 49% during the flowering and vegetative phases (Table 2).

Table.1 Proline content ($\mu\text{mol g}^{-1}\text{FW}$) in *P. vulgaris* (L.) plant leaves as influenced by water stress periods and rhizobial inoculation in field experiment for two consecutive seasons

1 st Season		2 nd Season		
Growth Phases	Vegetative	Flowering	Vegetative	Flowering
Treatments inoculation				
R+	4.39±0.31a	5.65±0.29a	4.96±0.25a	5.70±0.43a
R-	4.36±0.23a	4.95±0.28b	4.57±0.24b	5.65±0.55a
Stress Levels				
StrL1	3.45±0.15b	4.02±0.23b	3.81±0.11b	3.88±0.18b
StrL 2/StrL 3	5.30±0.25a	6.58±0.10a	5.72±0.22a	7.47±0.48a
Varieties				
Vrty 1	3.82±0.32c	4.80±0.53b	4.06±0.35b	4.58±0.57c
Vrty 2	3.63±0.38c	4.87±0.46b	4.42±0.30b	4.55±0.41c
Vrty 3	4.16±0.26bc	4.89±0.47b	4.32±0.25b	3.97±0.45c
Vrty 4	4.58±0.25b	6.12±0.35a	5.69±0.45a	6.43±0.90b
Vrty 5	5.69±0.59a	5.83±0.40a	5.32±0.37a	7.84±0.97a
3-Way Anova (F-Statistics)				
Rhz	0.02ns	17.24***	5.58*	0.02ns
StrL	67.67***	227.86***	135.86***	88.80***
Vrty	10.50***	10.80***	14.83***	11.33***
Rhz*StrL	0.004ns	2.23ns	0.87ns	1.54ns
Rhz*Vrty	1.03ns	0.87ns	0.35ns	0.38ns
StrL*Vrty	1.37ns	3.06*	4.15**	3.48*
Rhz*StrL*Vrty	2.11ns	1.50ns	0.34ns	0.52ns

+R: With *R. leguminosarum*, -R: Without *R. leguminosarum*; StrL 1: No water stress. StrL 2: Water stress at Vegetative Stage. StrL 3: Water stress at Flowering Stage. Vrty 1: KAT B9. Vrty 2: KAT B1. Vrty 3: F9 Kidney Selection. Vrty 4: F8 Drought Line. Vrty 5: JESCA. Values presented are means ± SE. *, **, *** = significant at $p \leq 0.05$, at $p \leq 0.01$ and at $p \leq 0.001$ respectively, ns = Not significant. Means followed by similar letter(s) in a given column are not significantly difference from each other at $p = 0.05$.

Table.2 Proline content ($\mu\text{mol g}^{-1}\text{FW}$) in *P. vulgaris* (L.) plant leaves as influenced by water stress periods and rhizobial inoculation in the screen house

Growth Phases	Vegetative	Flowering
Treatments inoculation		
R+	4.60±0.47a	5.20±0.49a
R-	3.03±0.43b	3.57±0.42b
Stress levels		
StrL 1	2.98±0.43b	2.98±0.43b
StrL 2/StrL 3	4.66±0.46a	5.79±0.41a
Varieties		
Vrty 1	2.62±0.71b	3.72±0.89a
Vrty 2	5.34±0.76a	5.13±0.61a
Vrty 3	2.99±0.67b	4.69±0.74a
Vrty 4	4.08±0.70ab	4.09±0.77a
Vrty 5	4.06±0.71ab	4.27±0.73a
3-Way Anova (F-Statistics)		
Rhz	7.80**	8.29**
StrL	8.97**	24.58***
Vrty	2.87*	0.75ns
Rhz*StrL	0.18ns	0.09ns
Rhz*Vrty	0.70ns	1.07ns
StrL*Vrty	0.69ns	0.48ns
Rhz*StrL*Vrty	3.27*	1.63ns

+R: With *R. leguminosarum*; -R: Without *R. leguminosarum*, StrL 1: No water stress. StrL 2: Water stress at Vegetative Stage. StrL 3: Water stress at Flowering Stage. Vrty 1: KAT B9. Vrty 2: KAT B1. Vrty 3: F9 Kidney Selection. Vrty 4: F8 Drought Line. Vrty 5: JESCA. Values presented are means \pm SE. *, **, *** = significant at $p \leq 0.05$ at $p \leq 0.01$ and at $p \leq 0.001$ respectively, ns = Not significant. Means followed by similar letter(s) in a given column are not significantly difference from each other at $p = 0.05$.

Fig.1 Interactive effects of stress level and five (5) *P. vulgaris* (L.) on proline content ($\mu\text{mol g}^{-1}\text{FW}$) in season (1) field experiment at flowering stage, StrL 2-: Control, StrL 3-: Water stress at flowering stage, Vrty 1-: KAT B9, Vrty 2-: KAT B1, Vrty 3-: F9 Kidney Selection, Vrty 4-: F8 Drought Line, Vrty 5-: JESCA)

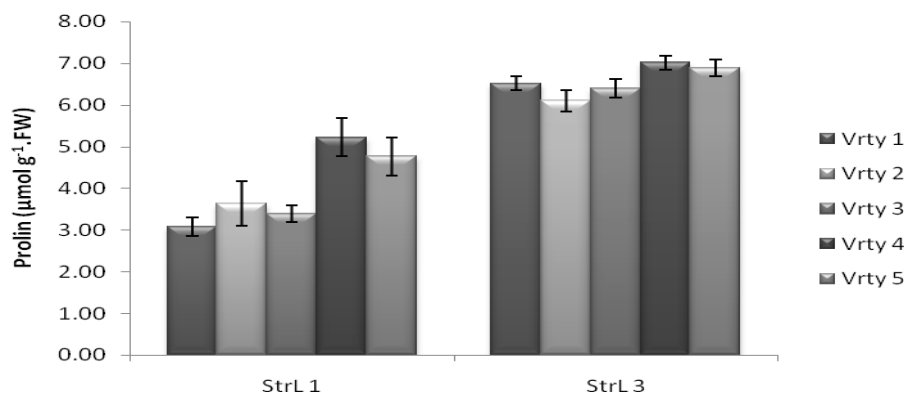


Fig.2 Interactive effects of stress level and five (5) *P. vulgaris* L. on proline content ($\mu\text{mol g}^{-1}\text{.FW}$) in season (2) field experiment at vegetative stage, StrL 1-: Control, StrL 2-: Water stress at vegetative stage, Vrty 1-: KAT B9, Vrty 2-: KAT B1, Vrty 3-: F9 Kidney Selection, Vrty 4-: F8 Drought Line, Vrty 5-: JESCA)

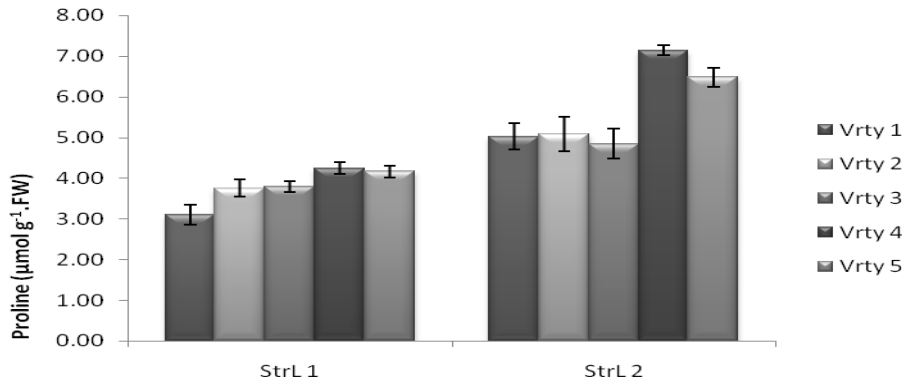


Fig.3 Interactive effects of stress level and five (5) *P. vulgaris* (L.) on proline content ($\mu\text{mol g}^{-1}\text{.FW}$) in season (2) field experiment at flowering stage, StrL 1-: Control, StrL 3-: Water stress at flowering stage, Vrty 1-: KAT B9, Vrty 2-: KAT B1, Vrty 3-: F9 Kidney Selection, Vrty 4-: F8 Drought Line, Vrty 5-: JESCA)

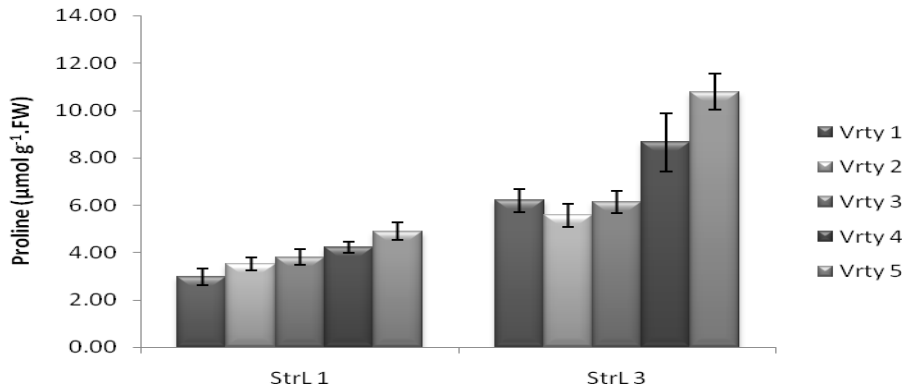
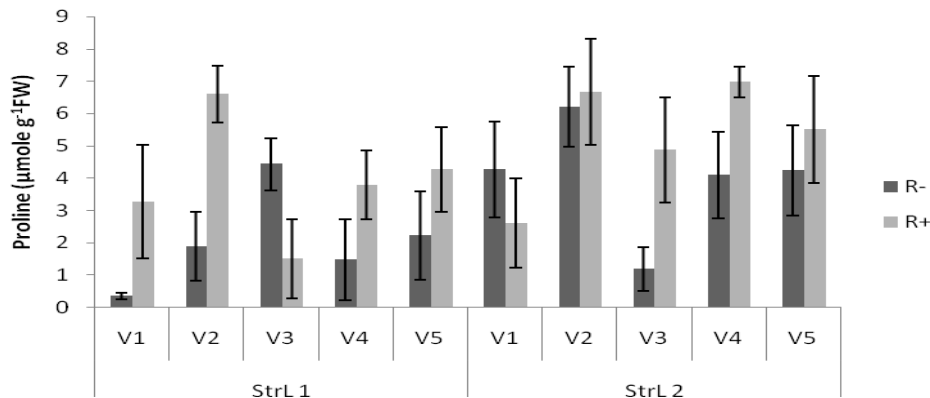


Fig.4 Interactive effects of rhizobial inoculation, stress level and five (5) *P. vulgaris* (L.) on proline content ($\mu\text{mol g}^{-1}\text{.FW}$) screen house experiment at vegetative stage, R--: Without rhizobial inoculation, R+-: With rhizobial inoculation, StrL 1-: Control, StrL -: Water stress at vegetative stage, V1=Vrty1-: KAT B9, V2=Vrty 2-: KAT B1, V3=Vrty 3-: F9 Kidney Selection, V4=Vrty 4-: F8 Drought Line, V5=Vrty 5-: JESCA)



Significant increase in proline content ($\mu\text{mol g}^{-1}\text{FW}$) was also recorded in variety 4 (*F8 Drought Line*) and 5 (*JESCA*) in field experiment, in season 1 and 2 respectively (Table 1). However, in the screen house experiment the proline content in bean varieties was as follows; *KAT BI* > *F8 Drought Line* > *JESCA* > *F8 Kidney Selection* > *KAT B9* (Table 2).

Interactive effects of inoculation with *R. leguminosarum biovar phaseoli* and stress period on proline content in selected *P. vulgaris* (L.) varieties

In the field experiments, there was significant interaction between stress levels and variety in proline content ($\mu\text{mol g}^{-1}\text{FW}$) (Fig. 1, 2 & 3). However, significant interaction was observed in the screen house between rhizobial inoculation, stress and bean varieties during the vegetative stage (Figure 4). Generally, the water stressed and rhizobial inoculated treatments had increased proline.

Rhizobial inoculation significantly improved proline content ($\mu\text{mol g}^{-1}\text{FW}$) of *P. vulgaris* (L.) as compared with non-inoculated treatment. Studies by other researchers (Daniel *et al.*, 2007; Djibril *et al.*, 2005; Kirda *et al.*, 1989; Ramos *et al.*, 2005; Sassi-Aydi and Abdelly, 2012) have also reported elevated level of proline under condition of sufficient N in which the proline levels increased in the tissues due to the action of ornithine pathway in enhancing proline synthesis, over the glutamine pathway (Sánchez *et al.*, 2002). Elbouthiri *et al.*, (2010) reported that *Rhizobium* inoculation in alfalfa resulted in highest leaf proline levels. Another study by Kohl *et al.*, (1991) in *Glycine max* plants inoculated with *Bradyrhizobium japonicum* showed higher amounts of proline in their tissues similar to what was found in this study. There was significant increase in proline content ($\mu\text{mol g}^{-1}\text{FW}$) in water stress treatment as compared

with un-stressed water treatment. Research evidence has shown that proline is commonly produced in higher plants and generally accumulates in large extent in response to environmental stresses such as water stress and /or drought (Ashraf and Foolad, 2007; Kapuya *et al.*, 1995; Lobato *et al.*, 2008; Siddiqui *et al.*, 2015; Tatar and Gevrek, 2008) and hence serving as a bio indicator of resistance or tolerance to water deficit (Bates *et al.*, 1973). In a closely related study, Sanchez *et al.*, (1998) reported increased proline content in pea plants subjected to drought stress.

Variety 4 (*F8 Drought line*), 5 (*JESCA*) and 2 (*KAT BI*) significantly increased proline content ($\mu\text{mol g}^{-1}\text{FW}$) of *P. vulgaris* L. in field and screen house experiment as compared with the other studied varieties. It has been established that accumulation of proline in plant tissues has been used as a biomarker and a parameter of choice for water stress tolerance in plants. This is due to the fact that water stressed plants produce proline as an adaptive and survival mechanism under water stress conditions (Chiang and Dandekar, 1995; Farooq *et al.*, 2009; Ford, 1984; Hayat *et al.*, 2012; Jaleel *et al.*, 2007; Masoudi-Sadaghiani *et al.*, 2011; Verbruggen and Hermans, 2008). The significantly higher amount of proline in variety 4 (*F8 Drought line*), 5 (*JESCA*) and 2 (*KAT BI*) suggests the potential of involving them in more advanced studies related to drought. Furthermore, the interactive effects between rhizobial inoculation, water stress and variety 4 (*F8 Drought line*), 5 (*JESCA*) and 2 (*KAT BI*) in producing elevated levels of proline is an indication which may warrant further studies.

It can be concluded from this study, rhizobial inoculation and water stress increased proline content in *P. vulgaris* (L.) Furthermore, the proline content was higher in varieties number 4 (*F8 Drought line*), 5 (*JESCA*) and 2 (*KAT BI*) and hence indicating their potential

to tolerate drought. Interactive effects between rhizobial inoculation, water stress and few identified varieties in enhancing the proline levels in the plants is an indication of various factors which may play a significant role in developing appropriate technology related to water stress tolerance in *P. vulgaris*.

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