First insight of exogenous addition of proline and glycinebetaine to mitigate fluorine toxicity effects on common bean seedlings

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Highlights

- *Exogenous glycinebetaine and proline were able to counteract F inhibitory effects on Phaseolus vulgaris seedlings growth.*
- *Sodium fluoride (NaF) showed more toxic effect than potassium fluoride (KF) on Phaseolus vulgaris seedlings growth.*
- *Proline was more effective than glycinebetaine in alleviating F toxicity effects on seedlings growth.*

Abstract

To counteract fluoride (F) stress-induced adverse effects on plants, one approach is the application of exogenous potential osmoprotectants such as proline (Pro) and glycinebetaine (GB). This experiment was carried out to evaluate the effects of exogenous application of Pro and GB on germination and growth of common bean seeds after potassium fluoride (KF) and sodium fluoride (NaF) exposure. The study was replicated under two different temperatures: the normal temperature required for seeds growth (20°C) and a higher (25°C) favourable for bean cultivation in Africa, the region most polluted with F. The results indicated that the beneficial effects of external supply of Pro and GB varied depending on temperature and the source of salt stress. NaF showed a more toxic effect than KF on growth of *Phaseolus vulgaris* seeds. Overall, Pro was more effective than GB to alleviate the undesirable effect of salt stress on morphological attributes under NaF stress (improving by 50% and 39% the length and the number of lateral roots), and especially at higher temperature the recovery role of Pro and GB was more relevant reaching the same value found under control conditions for the length of hypocotyl (3.28 cm).

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Introduction

Soil and ground water contamination with fluoride (F) is a serious problem in many countries with severe implications for human health. World Health Organization (WHO) established the safe limit of 1.5 mg L^{-1} in drinking water (Banerjee and Roychoudhry, 2019a). However, in several countries (America, Asia, Middle East and Africa), the F content in drinking water largely exceeds the permissible value. Furthermore, there is not a threshold limit value for F content in soil and plants.

In plants, the endogenous F level approximately increases of 3 ppm every 100 ppm in soil, up to about 2000 ppm level as reported by Bharti *et al* (2017). F gradually accumulates in plant tissues with time and when it reaches high level induces physiological and growth disorders, such as inhibition of germination and reduction of plant growth and biomass accumulation (Yadu *et al.*, 2016).

From a physiological point of view, the most frequent effects associated to F toxicity are photosynthesis alteration, nitrogen assimilation decrease, presence of chlorosis, chlorophyll disruption, alteration of enzyme activities (ribulose 1,5-biphosphate carboxylase; protein synthesis) (Panda, 2015). These symptoms converge in a general oxidative stress condition with an overproduction of reactive oxygen species (ROS) (Debska *et al*., 2012).

Besides, F toxicity is related to its permeability in plant tissues. F is absorbed by roots chloride channels and translocated in the whole plant through the xylematic flow (Banerjee and Roychoudhury, 2019a, 2019b). Once reached the plant cell, F might reduce the plasm membrane H+/ATPase activity causing the activation of abiotic signals cascade.

Plant stress symptoms associated with F toxicity are shown gradually and tend to be more severe under protracted exposure (Hong *et al*., 2016).

Soil and water are the principal source of F that is then accumulated in plant tissues and in edible parts, determining a potential risk for the entire food chains (Mondal and Gupta, 2015).

Among the different responses of plant to salts stresses, the most common is the overproduction of different type of compatible solutes (proline, glycine betaine, sorbitol, mannitol, *etc.*) (Ashraf and Foolard, 2007). The accumulation of osmolytes acts as membrane stabilizer and represents a temporary source of carbon and nitrogen that finally detoxicates plant cell from free radicals (Chandrakar *et al*., 2016).

Indeed, F toxicity in plant could be potentially mitigated screening candidate compounds, acting as osmolytes, that can be provided exogenously. Several studies revealed a key role of proline (Pro) and glycinebetaine (GB) in enhancing plants' tolerance to NaCl stress. In particular, the exogenous application of Pro in rice and lupin (Nounjan *et al*., 2012; Rady *et al*., 2015) revealed a reduction of stress symptoms in treated plants. Similar observations have been reported on the role of exogenous GB in enhancing the abiotic stress tolerance in different crops as well as *Phaseolus vulgaris* L. (Annunziata *et al*., 2019; Sofy *et al*., 2020).

Despite the promising role of these two osmolytes in mitigating the salts stress in plants, few information is available on their potential role in mitigating F stress. It has been shown that GB has a role in fluoride toxicity recovery in *Cajanus cajan* L. (Yadu *et al*., 2017), but no evidences are available for proline.

Common bean (*Phaseolus vulgaris* L.) is a grain legume extensively grown and consumed all over the world and represents one of the main food sources and sources of income of smallholder's farmers in Africa and Latin America (Beepe *et al*., 2012; Binagwa *et al*., 2018). These areas are already listed by the WHO as countries suffering an endemic fluorosis. The most important bean production area in eastern and southern Africa have a mean temperature (15-23°C) favourable for bean growth (Wortmann *et al*., 1998). Beans in the Eastern Kenya, central America, central Brazil and south Africa are produced at relatively high temperature (Wortmann *et al*., 1998; Beebe *et al*., 2012).

Considering the abiotic stress mitigation properties of GB and Pro, current study was aimed to evaluate their recovery efficacy on germination response and biometric traits of bean seedling under two salts treatments (NaF and KF) and two different temperature conditions (20°C, 25°C).

Materials and methods

Plant material and treatments

Seeds of *Phaseolus vulgaris* L., Borlotto nano "BOR", were placed in petri dishes under constant temperature conditions: at 25°C (Experiment 1) and at 20°C (Experiment 2).

For both experiments, seeds were treated as follow: i) Control (F0) and no osmolytes (C): 0 ppm salt + 0 µM osmolyte; ii) Control (F0) and GB: 0 ppm salt + 75 µM GB; iii) Control (F0) and Pro: 0 ppm salt + 10 mmol Pro; iv) Potassium fluoride (KF) and no osmolytes (C): 200 ppm KF + 0 µM osmolyte; v) KF and GB: 200 ppm KF + 75 µM GB; vi) KF and Pro: 200 ppm KF + 10 mmol Pro; vii) Sodium fluoride (NaF) and no osmolytes (C): 200 ppm NaF + 0 µM osmolyte; viii) NaF and GB: 200 ppm NaF + 75 µM GB; ix) NaF and Pro: 200 ppm NaF + 10 mmol Pro.

The treatment with salts was applied through soaking the seeds in solutions of deionized water (treatments 1, 2, 3) or a solution of KF 200 ppm (treatments 4, 5, 6) or a solution of NaF 200 ppm (treatments 7, 8, 9) for 6 h.

The tested concentrations of osmolytes were selected after a preliminary experiment in which four different concentrations of both osmolytes were compared.

Indeed, in the preliminary investigation, various concentrations of GB, *i.e.* 25, 50, 75 and 100 µM (following the procedure of Yadu *et al*., 2017) and Pro *i.e.* 3, 10, 20 and 30 mmol (based on Demiİr and Kocaçalişkan, 2001 under NaCl treatment) were tested for mitigation of F stress on the growth of *P. vulgaris* L. The results showed that the addition of 25, 50, 75 and 100 µM GB caused upsurge in lateral root length (LLR) by 161%, 150%, 188% and 154% compared to F stressed seedlings, respectively; while it decreased the main root length by 67%, 60%, 25% and 48%, respectively. Therefore, 75 µM of GB was used in this study, which showed maximum tolerance (highest increase of LR (188 %) and lowest decrease of the main root length (25%) to F-toxicity. For Pro, the addition of 3, 10, 20 and 30 mmol increased the LLR by 117%, 176%, 144% and 152% compared to F stressed seedlings; while it decreased the main root length by 60%, 44%, 47% and 46%, respectively. Hence, 10mmol of Pro was used in this study which showed maximum tolerance (176% of LLR and 54% of the main root length).

Out of these, the best tolerated concentration for each osmolyte was selected for the complete experiments.

According to the above-presented experimental design, the osmolytes were applied through irrigation (3 ml every 30 h).

Germination parameters and biometric traits

Over the 7-day-experiment, germinated seeds were recorded daily. From these records, both mean germination percentage (MG) and mean coefficient of velocity of germination (MCVG) were determined. MG was calculated as:

$$MG = \frac{\sum_{i=1}^{k} n_i}{N} \times 100 \qquad (1)$$

where,
n_i = number of seeds germinated at interval i.
N = total number of seeds germinated

The mean coefficient of velocity of germination (MCVG) was obtained as:

$$MCVG = \frac{\sum_{i=1}^{k} n_i t_i}{\sum_{i=1}^{k} n_i} \times 100 \qquad (2)$$

where,
n_i = number of seeds germinated at interval i.
t_i = time interval corresponding to n_i.

For both experiments, for each germinated seedling, the following biometric parameters were registered: main root length (LR), hypocotyl length (LH), number of lateral root (NLR), length of lateral root (LLR), fresh weight (FW).

Statistical analyses

The experiments were laid out with a full factorial design with 3 replicates for each treatment. Each experiment was replicated twice.

Statistical analyses were performed in RStudio application of R software (R Core Team, 2014) environment (packages lme4, emmeans, multicomp). The examined biometric traits, after having checked the main assumption of normality and homoscedasticity, were processed using a linear model in a two-ways ANOVA.

MG and MCVG were processed using a generalized linear model with a quasi-binomial distribution using a logit link function.

The significance of differences between treatment means was evaluated by Tukey's test at $P<0.05$.

Results and discussion

The present study explored the mitigation effects of two osmolytes (GB and Pro) on some biometric and germination parameters recorded on *P. vulgaris* seeds grown under two different temperature conditions (20°C and 25°C) and stressed by two F sources (KF and NaF).

The results of two-ways ANOVA for both experiments are reported in Table 1. In both cases, the growth and development of *P. vulgaris* seedlings were negatively affected by both F sources, but exogenous addition of GB and Pro unveiled enhancement in germination and biometric parameters (Table 1; Figure 1).

Experiment #1

At higher temperature (25°C, experiment 1, Exp 1), the two sources of F dramatically reduced the germination percentage (MG), from 75% (F0) to 0% (KF and NaF 200 ppm) (Figure 1A). The exogenous application of GB significantly increased MG both in control condition and under KF treatment, while Pro enhanced significantly MG in all the tested conditions.

The mean coefficient of velocity of germination (MCVG) showed the same significance of MG depending on salts and osmolytes, which highlighted the role of Pro addition to fasten the germination process in all the treatments and to mitigate the toxicity effect due to salts. GB addition was able to replicate the same performance already discussed for Pro only under control and KF treatment (Figure 1B). These results were partially in disagreement

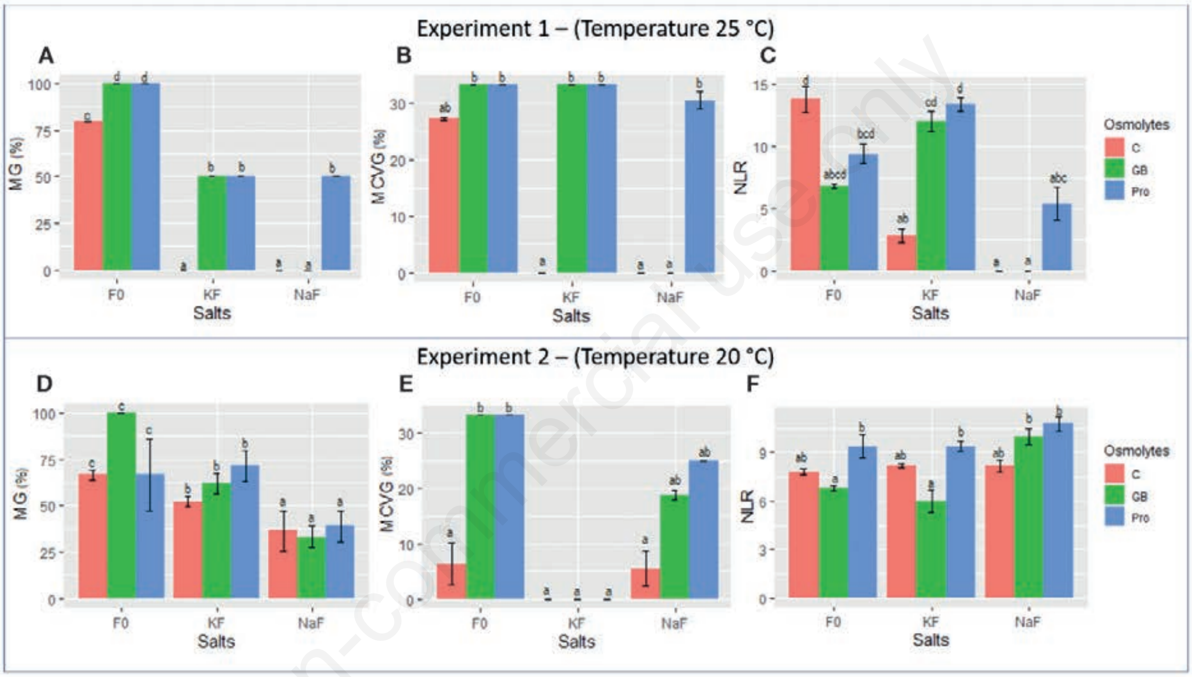

Figure 1. Mean germination percentage (MG), mean coefficient of velocity of germination (MCVG) and number of lateral roots (NLR) under three salt levels: 0, KF (200 ppm) and NaF (200 ppm) and supplied with three levels of osmolytes: control (C, no osmolytes), glycinbetaine (GB: 75 μM), proline (Pro: 10 mmol) tested at 25°C (A, B, C) and at 20°C (D, E, F). Vertical bars represent standard error. Different combinations of lower case letters indicate significantly differing means (P<0.05, Tukey's test).

Table 1. Significance of the effects of salts, osmolytes and their interaction on biometric and germination parameters.

	LR	LLR	NLR	FW	LH	MG	MCVG
Experiment #1							
Salts	***	***	***	***	***	***	***
Osmolytes			**	***		***	***
Salts × osmolytes			***	***	**	***	***
Experiment #2							
Salts	**	***		***	***	*	***
Osmolytes	**	***	*	***	*		***
Salts × osmolytes		***		***			

LR, root length; LLR, length of lateral root; NLR, number of later roots; FW, fresh weight of seedlings; LH, length of hypocotyl; (MG, mean germination percentage; MCVG, mean coefficient of velocity of germination. *P≤0.05; **P≤0.01; ***P≤0.001.

with a previous study conducted by Yadu *et al*. (2017) which confirmed that GB significantly alleviated the inhibition in germination and overall growth responses induced by F in *Cajanus cajan* L. The apparent discrepancy could be associate to the high level of NaF used (200 ppm) in this research compared to 75 ppm reported by the authors and the quantity of exogenous GB applied, beyond the test species which were different.

However, our results regarding the Pro effect are confirmed by several research studies highlighting that exogenous application of Pro to plants increase their stress tolerance, providing osmo-protection and promoting bean growth under KF and NaF stressed conditions (Dar *et al.*, 2016).

Beside seed germination, F also affected the seedling growth (data not shown). Data witnessed that F drastically reduced root length (LR) and hypocotyl length (LH) by 100% and 75%-64% under KF and NaF treatment compared to the control, respectively. As previously reported, GB and Pro, under abiotic stress condition, increase the rate of cell division and elongation, and improves the plant growth (Luo *et al*. 2015). This was not confirmed in our study, where seedlings growth was partially inhibited after the addition of GB and Pro under F stress (data not shown). Without salts application, GB and Pro enhanced both LH and LR up to values recorded for the control (data not shown).

In this study, lateral roots were monitored as well. Length and number of lateral roots (LLR; NLR) significantly decreased as response to salts stress (Table 1). In general, the addition of Pro induced a recovery of about 10% and 50% of LLR in plant treated with KF and NaF, respectively. No effect was observed after GB application (data not shown). Furthermore, NLR was significantly affected by F addition with specific osmolyte response (Table 1; Figure 1C). Without salt, the highest NLR was observed in F0 (13.80) (Figure 1C), while the addition of GB and Pro reduced NLR (6.80 and 9.40 respectively). Under F, a significant reduction of NLR was detected compared to control condition (2.80 and 0 for KF and NaF, respectively). The addition of osmolytes deeply impacted the NLR with a salt specific response. Under KF treatment, Pro induced a complete recovery of NLR, obtaining a quite same value recorded in F0 (13.40); while GB showed an almost complete restoration of NLR value (12.0). Under NaF treatment, the addition of GB did not have effect on NLR recovery, while Pro addition was able to recover about 39% of NLR.

Experiment #2

When the experiment was performed at lower temperature (20°C; Experiment 2, Exp2), osmolytes and salts showed different effects on germination and seedlings growth compared to the Exp1. These differences might be associated to the combined effect of higher temperature and salinity for Exp1 and to the only salts effect in Exp2.

Overall, the addition of F induced a significant reduction of MG (Table 1; Figure 1D) compared to the control. In F0, the addition of GB increased MG from 70% (F0) to the maximum value of 100%, while Pro kept the same germination value observed in F0. A significant reduction of MG has been induced after the addition of KF (approx. 50%) and NaF (approx. 30%). Indeed, the osmolytes addition showed more effectiveness under KF treatment than under NaF. In particular, both osmolytes added to KF completely restored MG to F0 value, while under NaF a teeny improvement was observed.

The effect of GB and Pro was more evident on MCVG. This parameter, without salts, increased six times after osmolytes addition. Differently, it remained stable and equal to zero under KF treatment, while it changed with Pro and GB addition under NaF treatment, underlying the importance of adding osmolytes in condition of high salt stress to fasten germination process (Figure 1E). At lower temperature both salts halved LR (data not shown). Under KF treatment, Pro completely restored LR, even exceeding LR values recorded under F0 treatment, while GB showed a limited effect with almost no difference with the KF treatment (data not shown). Under NaF, the two osmolytes showed a different trend. GB demonstrated a better recovery activity compared to Pro. Indeed, Pro application enhanced LR reaching values closed to F0 condition, while LR obtained with GB application overcame those values.

LH increased significantly with osmolytes addition while it was significantly reduced by salts treatment (Table 1). KF and NaF reduced LH by 76% and 67%, respectively. The addition of both osmolytes induced a partial recovery of LH, highlighting a better performance of Pro compared to GB. About LH, the best recovery was found in NaF+ Pro (2.82 cm) thus approaching the average value found under control condition (F0: 3.28 cm). As reported in Exp1, also in Exp2 length and number of lateral roots (LLR, NLR) significantly decreased as response to salts stress (Table 1). In general, the addition of Pro induced a recovery of about 50% and 60% of LLR in plant treated with KF and NaF, respectively. GB addition provoked halved effect on LLR compared to GB addition for each tested salt (data not shown).

The effect of F was not significant in terms of NLR (Table 1). Conversely, the addition of Pro was more promising than GB for all salt treatments (Figure 1F).

The literature proved that exogenously application of GB and Pro improved growth and other physiological attributes of plants exposed to abiotic stress (Dar *et al.*, 2016).

GB mitigates adverse effects of salinity stress in different plant species. Indeed, Lutts (2000) reported that foliar application of GB significantly improved salt tolerance in rice plants, while Ashraf and Foolad (2007) reported that exogenous application of GB on tomato plants cultivated under salinity stress increase of about 40% their fruit yield compared with untreated plants. Moreover, under salt stress, GB may also indirectly protect cells from environmental stresses *via* its function in signal transduction, thanks to its role in Na^+/K^+ discrimination, which substantially or partially contributes to plant salt tolerance ((Dar *et al.*, 2016). However, a better understanding of GB mechanism of action and the magnitude of its effects on different plant species would be essential to implement its application.

Exogenously applied Pro plays also an important role in enhancing plant stress tolerance. Pro can protect cell membranes from various environmental stresses by enhancing activities of antioxidants. An involvement of Pro in developmental processes, seed germination, flower transition, flower development and other developmental processes have been reported by Dar *et al.*, (2016) but further research is necessary to better identify its role against abiotic stresses.

Conclusions

The results of this study, for the first time, described the specific role of Pro and GB in improving F stress tolerance in *P. vulgaris* at early stage of plant development.

Even though limitations exist for extrapolating results to the field scale, such as the study was focused on the very early stage of development of *Phaseolus vulgaris,* we tried to accurately quantify the effects of exogenous applications of GB and Pro to bean seeds under F stress, on germination parameters and seedling biometry in order to draw a clear idea of what happens in the period immediately following germination.

In this phase, the beneficial effects of external supply of osmoprotectants changed depending on temperature and the severity of salt stress. In this study, Pro was more effective than GB in alleviating the undesirable effects of F stress on some morphological attributes caused by NaF.

Therefore, for effective application and commercial use of exogenous GB and Pro as biostimulants of crop to reach F stress tolerance, the mechanisms of actions of these compounds, the optimal concentrations depending on the susceptibility of the species and the specific salt stress condition, and the most appropriate plant developmental stage in which performing the application still need to be carefully determined.

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