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Polyamine Modulation by antagonistic bacteria *Bacillus subtilis* in chickpea during *Fusarium oxysporum* f. sp. *ciceri* interaction

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ABSTRACT

Chickpea (*Cicer arietinum* L.) is pivotal source of protein for vegetarian diet, however, its productivity is adversely affected by wilt disease. Non pathogenic rhizospheric microorganism's leads to induce resistance and are found to be effective in management of this disease. The polyamines (PAs) content and its metabolism are the key in plant microbial interaction, so the alteration in PAs *viz.* spermidine (SPD), spermine (SPM) and putresine (PTR) in chickpea by *Bacillus substilis* isolate K18 (*BS*-K18) effective antagonist (75%) of *Fusarium oxysporum f. sp. ciceri* (*Foc*) and having PGPR traits was analyzed under *Foc* stress. The higher PAs content was reported in resistant variety (WR-315) compared to susceptible variety (JG-62). The PTR was dominant PA present in chickpea, further overall root tissue reported higher PA content as compared to leaves tissue. The PA content was constitutively improved by *B. subtilis* seed treatment in resistant and susceptible varieties. The *Foc* stress leads to induction of PA content in leaves and root tissue, where its content was higher in resistant variety as compared to susceptible variety. The *BS-K*18 seed treatment under *Foc* stress leads to induction of PA content as compared to both treatments alone, the SPD and SPM were more induced in leaves and root tissue of susceptible variety whereas PTR was more induces in resistant variety. Overall, polyamines were induced up to 3 DAT then after decline suggest their early role in plant defence mechanism, further PTR was found to be dominating polyamine during chickpea-*Foc* interaction under *BS*-K18 treatment. The *Bacillus subtilis* seed treatment leads to improve wilt tolerance in susceptible var. JG-62 through modulation of PAs, the same mechanism also helped to enhanced effectiveness of resistant var. WR-315.

Key words: Chickpea, *Bacillus subtilis* isolate K18, *Fusarium oxysporum* f. sp. *Cicero,* Polyamine, UFLC.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is pivotal source of protein for vegetarian diet called poor men's meat; however the productivity of chickpea is far below its potential. *Fusarium* wilt caused by *Fusaruim oxysporum* f. sp. *ciceris* (Padwick) Matuo & K. Sato (*Foc*) is one of the most destructive vascular diseases of chickpea first reported from India by Butler in 1918 which causes yield loss up to 90% annually under epidemic condition (Chaube and Pundhir, 2005). Biological control of *Fusarium* wilt is a potential component of integrated disease management (IDM) where the principle of bacteria or fungal antagonist has been exploited for the control of disease. The application of microorganisms to control diseases, which is a form of biological control, is an environment friendly approach. Different scientists have realized the potential of rhizospheric microorganism in control of *Foc* in chickpea (Schmitdt *et al.*, 2006). As reported by many researchers, all natural resistance, induced resistance either by pathogen or by bio-control agent is under genetic control and genes are expressed through biological products such as secondary metabolites *viz.* phenolics, lignin, callose, suberin, phytoalexins, alkaloids, terpenes, glycosides and pathogenesis related proteins, all of which contributes to disease resistance (Gupta *et al.,* 2010; Wang *et al.,* 2007).

The aliphatic polycationic compound actively known as polyamines occur ubiquitously in the plant kingdom, together with their diamine precursor putrescine. These low molecular weight compounds are positively charged at physiological pH and because of this they are known to bind to negatively charged molecules, *e.g*. nucleic acids, acidic phospholipids and various types of proteins and by this means they affect the structure and function of these molecules (Cohen, 1998). In higher plants, polyamines occur in free form, bound electrostatically to negatively charged molecules and conjugated to small molecules and proteins. Polyamines have been implicated in a variety of plant growth and developmental processes involving cell proliferation and differentiation, morphogenesis, development and stress tolerance (Matto *et al*., 2010). Additionally, there is accumulating evidence that infections of various pathogenic fungi and viruses bring about dynamic changes in polyamine

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metabolism not only in the infected cells but also in other regions of the host plants. Polyamine-accumulating transgenic plants exhibited an increased tolerance levels to multiple abiotic stresses such as salinity, drought, low and high temperature and heavy-metal and resistance against fungal wilt disease caused by *Fusarium oxysporium* (Prabhavathi and Rajam, 2007).

There is a considerable amount of information about the role played by PAs during the interactions of plants with either pathogenic or beneficial microbes. These data indicate that changes in PA metabolism constitute a key adaptive response of the plant, and that their occurrence determines the development of the interaction. In turn, evidence suggests that some microorganisms are able to perturb plant PA metabolism in order to adjust it to their own requirements. Plant PA metabolism undergoes remarkably changes during plant– microbe interactions (Walters, 2003; Hussain *et al*., 2011). The roles played by PAs during these processes are considerably intricate. This is due to the fact that PAs are not only essential to maintain cell viability, but function as signaling molecules regulating many of the responses that help the plant to cope with biotic stress. The modulation of PA metabolism is so important for the outcome of the pathogenic interactions that not only the plant modifies PA concentration in response to pathogens, but some microorganisms have developed mechanisms to induce modifications of PA levels in host tissues. Therefore, it seems that when plants are attacked by pathogens, the organism that takes control of the PA machinery has a great opportunity to take the lead (Jimenez-Bremont *et al*., 2014). The interaction of plants with beneficial microorganisms also induces changes in PA metabolism, and the establishment of mutualism with symbionts such as Rhizobia and Mycorrhizae appear to depend on PA levels. The information on rhizhosperic microorganism mediated alteration in plant PAs upon pathogen infection is scare and hence in present study the antagonist plant growth promoting rhizospheric (PGPR) strain of *Bacillus subtilis* isolate K18 induced modulation of polyamines in chickpea were studied under *Fusarium oxysporium* f. sp. *ciceri* stress.

MATERIALS AND METHODS

Plant material

The seeds of resistant (WR-315) and susceptible (JG-62) chickpea variety were procured from International Crops Research Institute for the Semi Arid Tropics (ICRISAT) Patancheru, Andhra Pradesh, India was used in present study. The healthy and uniform seeds were surface sterilized with 0.01 % mercuric chloride ($HgCl_2$) for 1 min followed by rinsing with autoclaved double distilled water (DDW) four times, to remove all traces of $HgCl_2$. The surface sterilized seeds were used in further study.

PGPR isolate

The highly effective antagonist *Bacillus subtilis* isolate K18 (*BS*-K18) against *Fusarium oxysporum* f. sp. *ciceris* found in previous study where it reported 75% inhibition of *Foc* under *in vitro* condition was used in present study (Suthar *et al*., 2017). It was preserved on agar slant at 4°C and glycerol stocks were prepared for long term preservation of isolates.

Seed treatment

The overnight grown culture of *BS*-K18 in Nutrient Broth (NB) was used for seed treatment of surface sterilized seeds at the rate of 10 ml kg^{-1}. The treated seeds were dried under shed and then, sown in sterile sand.

Foc stress

The *Foc* sick soil was prepared by mixing field soil and farm yard manure (FYM) in the proportion of 1:1 and sterilized in

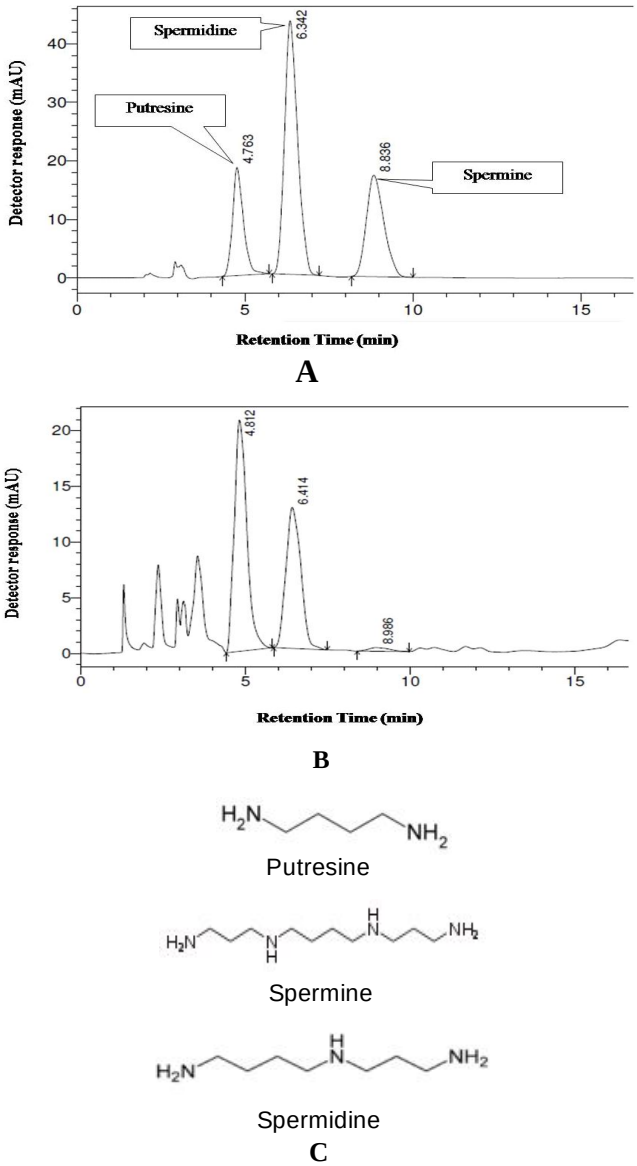


Fig 1: A. Chromatograph of polyamines standard separated by UFLC visualised at 210 nm; **B.** UFLC chromatograph of polya mines for root tissue of WR_{315} SiDi treatment at 7 DAT; **C.** Chemical structure of polyamies standard used in present study.

autoclave. Sorghum grains inoculated with pathogen *Fusarium oxysporum* f. sp. *ciceri* which had microbial load 2.5 X 10^7 cfu g^{-1} in sorghum grain media was then added to the soil in the proportion of 1:10 (Sorghum grain Inoculums + Sterilized soil mixture). The bags were filled with these mixtures @ 2.5 kg per bag as a sick soil and were used to create *Foc* stress.

***In vitro* experiment**

The seeds were treated with *BS-K18* and allowed to grow for nine days on sterile medium. The nine day old seedlings were transferred to bags containing *Foc* sick soil for creating wilt stress and kept under a net house.

Polyamine analysis

The polyamines *viz.,* spermidine, spermine and putrecsine was analyzed using Ultra Fast Liquid Chromatography (UFLC; Shimadzu, Japan) equipped with PDA detector 2800 (Shimadzu; Japan) and C-18 column (Sethi *et al.,* 2011). The mobile phase based on an isocratic program which included 60% methanol as a solvents system was used where samples were run at a flow rate of 0.750 ml min^{-1} and visualized at 290 nm with a UV-visible detector (Fig 1). The PA content was analyzed from leaves and root tissue at 0, 3 and 7 days after transfer (DAT) to sick soil. The significance difference in data was analyzed using factorial completely randomized design (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

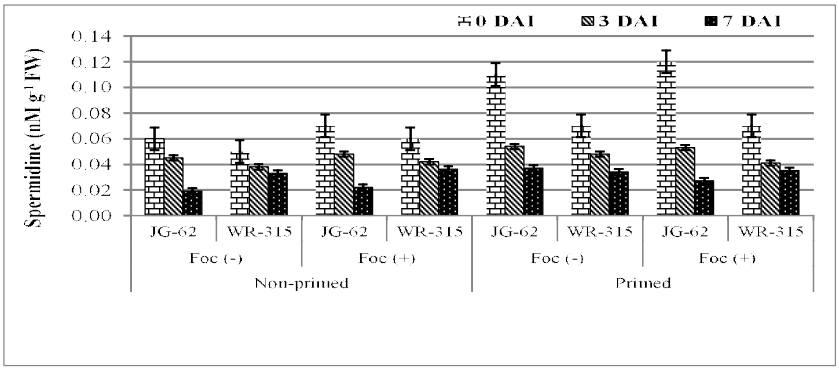
Polyamines are involved in normal cellular activity, apart from that they have been reported to have defence role during abiotic and biotic stress. The beneficial MO/pathogen infection leads to alteration in PAs and the PA metabolic enzymes (Hussain *et al.,* 2011). The *B. subtilis* seed treatment significantly increased PAs content in leaves and root tissue of both varieties at different time intervals. The PTR content was reported significantly higher as compared to SPD and SPM. The *Foc* stress leads to induction of PA content in leaves and root tissue, where higher PA content was found in resistant var. WR-315 as compared to susceptible var. JG-62. The *BS-K*18 seed treatment under *Foc* stress leads to induction of PA content as compared to both treatments alone, although the induction was more in susceptible variety.

The SPD content in leaves tissue was declined over the time, but *BS*-K18 treatment showed higher SPD content compared to respective control. In leaves tissue of susceptible variety *BS*-K18 treatment under *Foc* stress (SiDi) leads to 2.00 fold increase (0.120 nM g^{-1} FW) in SPD content as compared to 1.17 fold (0.070 nM g^{-1} FW) under *Foc* stress alone (SoDi) at 0 DAT. The higher content was reported at 3 and 7 DAT also for the same treatment. In case of resistant variety the *BS*-K18 under *Foc* stress (SiDi) leads to initially higher fold increase in SPD content as compared to *Foc* stress (SoDi) alone, but at 3 and 7 DAT it was reduced (Fig 2A). The SPD content was increased up to 3 DAT in leaves and root tissue under different treatment conditions in both varieties which found to be declined at 7 DAT. The *BS*-K18 leads to improve SPD content in root tissue of both varieties (SiDo), however its application under *Foc* stress reported lower SPD content as compared to *Foc* stress alone at different time intervals. Maximum SPD content in root tissue (0.040 nM g^{-1} FW) was reported in resistant variety under *Foc* stress alone (SoDi) at 3 DAT where as it was only 0.030 nM g^{-1} FW in *BS*-K18 under *Foc* stress (SiDi).

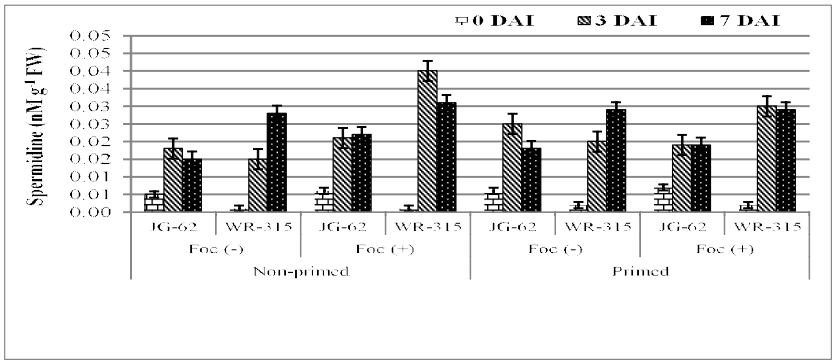
The resistant variety showed higher SPM content constitutively as well as under *Foc* stress as compared to sasceptible variety, however *BS*-K18 treatment under *Foc* stress helped to improve SPM content in susceptible variety. The SPM content was induced by *BS*-K18 seed treatment in leaves and root tissue of both varieties. In both tissues *BS*-K18 treatment under *Foc* stress (SiDi) leads to higher fold increase in SPM content as compared to *Foc* stress alone (SoDi) in susceptible variety than resistant variety at various time intervals. The *BS*-K18 treatment under *Foc* stress reported significantly higher SPM content, 0.586 and 0.325 nM g^{-1} FW in leaves tissue susceptible variety as compared to resistant variety where it was 0.432 and 0.315 at 3 and 7 DAT respectively (Fig 2B). The higher fold increased in SPM content in root tissue that is 1.11 and 1.30 fold was reported in susceptible variety for *BS*-K18 under *Foc* stress as against 1.04 and 1.03 fold increased in resistant variety at 3 and 7 DAT, respectively.

Higher PTR content was observed in root tissue as compared to leaves tissue in both varieties under different treatment conditions. The *BS*-K18 seed treatment leads to improve PTR content in leaves and root tissue of both verities. The PTR content was increased up to 3 DAT than after declined in leaves and root tissue of both varieties for all treatment condition. The *Foc* stress induced PTR content in leaves and root tissue of both varieties, however the induction was more in resistant variety. The *BS*-K18 treatment under *Foc* stress (SiDi) reported higher PTR content as compared to *Foc* stress alone (SoDi), maximum PTR content in root tissue (18.830 nM g^{-1} FW) and leaves tissue (2.615 nM g^{-1} FW) of *BS*-K18 treatment under *Foc* stress was observed in resistant variety at 3 DAT (SiDi; Fig 2C). Overall, the Interaction effect of variety, *B. subtilis* seed treatment and *Foc* stress on different polyamines *viz*. spermidine, spermine and putresine in leaves and root tissue of both varieties were found significant which propose the modulation of polyamines by antagonistic bacteria *Bacillus subtilis* in chickpea during *Fusarium oxysporum* f. sp. *ciceri* interaction.

Polyamines play important role in biotic stress tolerance where transgenic chickpea plants producing high level polyamine exhibited the increased tolerance levels to multiple abiotic stresses and wilt disease (Prabhavathi and Rajam, 2007).The accumulation of free PAs usually accompanied of a rise in conjugated PAs and leads to affect microbial growth and prevent development of disease in plant (Mackintosh *et al*., 1997; Walters *et al*., 2001). Cohen (1998) reported higher level of PTR compared to other PAs,

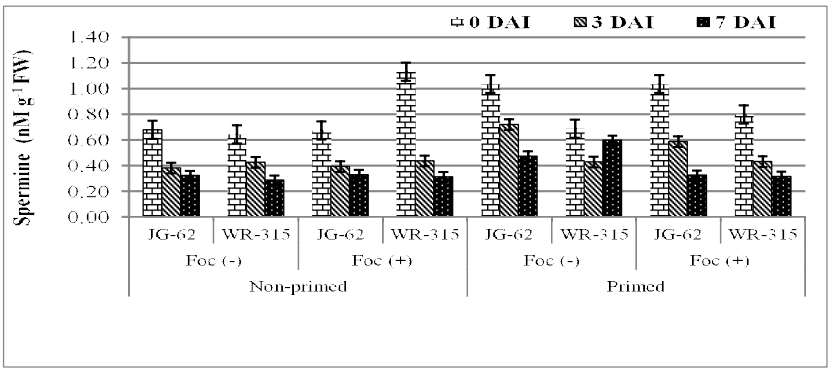


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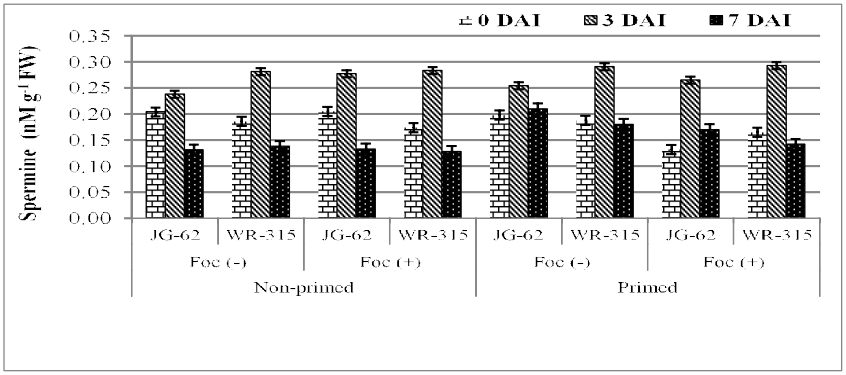


Root

Fig 2A

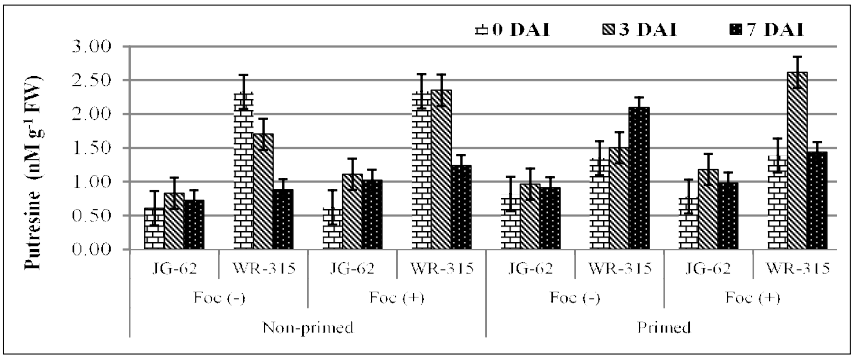


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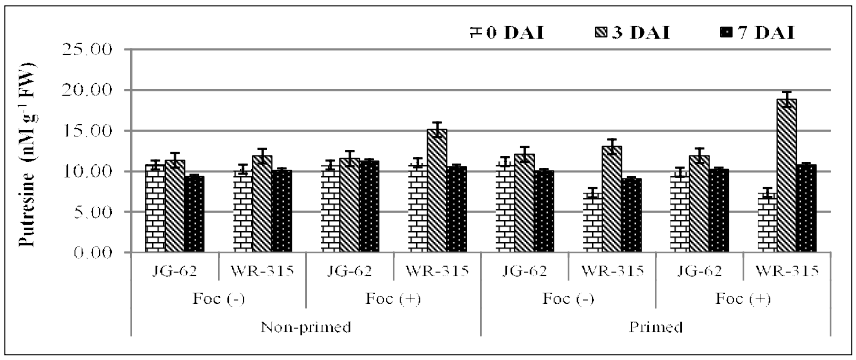


Root

Fig 2B



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Root

Fig 2C

Fig 2: A. Effect of *B. subtilis* isolate K18 on spermidine (SPD) content during *Foc* stress in chickpea.
B. Effect of *B. subtilis* isolate K18 on spermine (SPM) content during *Foc* stress in chickpea.
C. Effect of *B. subtilis* isolate K18 on putresine (PTR) content during *Foc* stress in chickpea.

Note: R: Resistant var. WR-315. **S:** Susceptible var. JG-62.
SoDo: Without *B. subtilis* seed treatment and normal soil transplanted. **SoDi:** Without *B. subtilis* seed treatment and wilt sick soil transplanted.
SiDo: *B. subtilis* seed treated and normal soil transplanted. **SiDi:** *B. subtilis* seed treated and wilt sick soil transplanted.

in present study also the higher level of PTR was observed. The concentration of PAs is under strict control in eukaryotic cell, as not only its depletion but also excessive accumulation is deteriouse (He *et al*., 1993). The higher PAs accumulation in resistant variety as compared to susceptible variety is reported by various researchers during their study on different plant-pathogen system (Cowley and Walters, 2002a; Asthir *et al*., 2004). The *BS*-K18 seed treatment helped chickpea plant to accumulate PAs during plant-*Foc* interaction, such evidence of enhanced PAs production by beneficial microorganism in plant are scare. Many studies reported induction of PAs during plant pathogen interaction. Angelini *et al.* (1993) observed the increased POX and DAO activities and putrescine level after *Ascochyta rabiei* infection in both resistant and susceptible chickpea cultivar as compared to control plants, with a greater enhancement of both enzyme activities and diamine level in resistant one. Cowley and Walters (2002) observed incompatible interaction between barley infected with powdery mildew fungus (*B. graminis*, *hordei*) increased the level of free putrescine, spermine and conjugated forms of putrescine, spermidine and spermine at 1-4 days following inoculation. Further work showed in an incompatible interaction between barley and powdery mildew, where the resistance was penetration based, levels of spermidine and putrescine were found to increase 1-3 day after inoculation (Cowley and Walters, 2002b). Mhaske *et al*. (2013) studied polyamine profiling during *Fusarium oxysporum* f. sp. *ricini*-castor interaction and proposed role of high titers of polyamines, in disease resistance possibly through HR induction.

The beneficial rhizhosperic microorganism, *Bacillus subtilis* isolate K18 seed treatment leads to induction of PAs in chickpea. This seed treatment also reported enhanced PAs production during chickpea *Foc* interaction which helped to both resistant and susceptible variety to alleviate *Foc* stress. This study show induces resistance against *Fusarium oxysporum* f. sp. *ciceri* in chickpea mediated through modulation of PAs by *Bacillus subtilis* seed treatment.

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