Endophytic Colonization of *Burkholderia phytofirmans* Strain PsJN Induces Drought-Stress Tolerance in Maize

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Abstract

Drought stress is one of the major constraints hampering agricultural production owing to its impact on plant water status and photosynthetic pigments. The effect of inoculation of a plant-growth promoting bacterium *Burkholderia phytofirmans* strain PsJN on growth, water status and photosynthetic activity of two maize cultivars under drought stress conditions was investigated. Drought stress induced by withholding irrigation had drastic effects on growth and photosynthesis of maize seedlings. However, seed bacterization of maize with *B. phytofirmans* PsJN improved plant (root/shoot) biomass, leaf chlorophyll contents and relative water status up to 61, 21 and 29%, respectively over control under drought stress conditions. Similarly, PsJN inoculation significantly increased photochemical efficiency of PSII and photosynthetic activity up to 9 and 68% of the cultivar 'Mazurka' compared to control under stressed conditions. Contrary to this, inoculation decreased electrolyte leakage compared to uninoculated seedlings under drought stress. The inoculant strain efficiently colonized maize seedling and recovered from root, shoot and leaves of irrigated and stressed plants. In conclusion, our study clearly demonstrates that bacterial inoculants could be used to minimize the negative effects of drought stress on growth and photosynthesis of maize.

INTRODUCTION

Drought is a potential major constraint to maize production in all areas where it is grown. Global warming, deforestation, and urbanization will all increase the severity and frequency of drought in the future, leading to a possible decrease in global food production at the same time that a steadily increasing human population which could hit nine billion by 2050 demands an increase in food supplies. In spite of limited arable land coupled with the rising consumer's demand of high quality food, free from chemicals, food production is one of the major global challenges. Therefore, it has become obligatory to investigate the ways to mitigate the adverse effect of drought stress and increase crop productivity within a finite natural resource basis.

During the past couple of decades, plant growth-promoting rhizobacteria (PGPR) have received worldwide importance and acceptance in agricultural practice and are promising alternatives to agrochemicals (fertilizers and pesticides). In the late 1970s Kloepper and Schroth introduced the term "plant growth promoting rhizobacteria (PGPR)" to describe bacteria that colonize plant roots after seed inoculation and that stimulate plant growth (Kloepper and Schroth, 1978). The PGPR are reported to influence the growth, yield, and nutrient uptake by an array of mechanisms. Some bacterial strains directly regulate plant physiology by mimicking synthesis of plant hormones, whereas others increase mineral and nitrogen availability in the soil as a way to augment growth (Yasmin et al., 2007).

Endophytes are per definition microorganisms – bacteria or fungi – that colonize living plant tissue without being pathogenic to the plant. Endophytic bacteria may in

future be even more important than rhizosphere bacteria in promoting plant growth because they escape competition with rhizosphere microorganisms and achieve more intimate contact with the plant tissues. In addition, inherent nature of certain endophytes to potentially colonize plants in a systematic manner provides a novel approach as a delivery system to plant for various beneficial traits (Döbereiner, 1992; Kobayashi and Palumbo, 2000; Fuentes-Ramirez and Caballero-Mellado, 2005).

Burkholderia phytofirmans strain PsJN is one of the best studied bacterial endophytes. Originally isolated from surface-sterilized *Glomus vesiculiferum*-infected onion roots (Frommel et al., 1991), strain PsJN has been shown to colonize a wide variety of plants (e.g., potato, tomato, peat moss and grapevines (Compant et al., 2008)) and that it stimulates plant growth and vitality in many of its host plants.

Therefore, the aim of this study was to investigate the effect of *B. phytofirmans* strain PsJN inoculation on growth, relative water status, chlorophyll fluorescence, and photosynthetic pigments of maize (*Zea mays*) under drought stress conditions.

MATERIALS AND METHODS

The effect of *Burkholderia phytofirmans* strain PsJN inoculation on the physiology and growth parameters of maize cultivars 'Mazurka' and 'Kaleo' was tested under greenhouse conditions. We used a genetic variant of the bacteria strain *B. phytofirmans* PsJN::gusA (Compant et al., 2005) that carries a beta-glucuronidase reporter gene (*gus*A) that allows specific visualization of bacterial cells upon color formation. Maize seeds were kindly provided by DOW AgroSciences Vertriebsges.mbH Neusiedl am See, Austria.

Greenhouse Experiment and Growth Conditions

Plants were grown in pots filled with local field soil. The soil was ground, passed through a 2 mm sieve and analyzed for various physico-chemical characteristics, i.e., sand, 32%; silt, 38%; clay, 30%; pH, 7.28; total carbon, 2.4%; total nitrogen, 0.23%; available phosphorus, 40 mg/100 g; extractable potassium, 19 mg/100 g soil. Each pot was filled with 15 kg soil receiving nutrient inputs of NPK at 160, 100, 60 kg ha^{-1}, respectively.

For seed inoculation, surface-sterilized seeds were treated with bacterial suspension of strain PsJN (10^8-10^9 CFU ml^{-1}) under lab conditions. In the case of uninoculated control, seeds were treated with sterilized LB broth. Inoculums were prepared by growing *Burkholderia phytofirmans* strain PsJN in 250 ml Erlenmeyer flasks containing LB media amended with spectinomycin (100 µg ml^{-1}). The broth was inoculated with a single cell and incubated at 28±2°C for 72 h in a shaking incubator (VWR International, GmbH, Austria) at 180 r min^{-1}. Five maize seeds either inoculated with strain PsJN or broth only were sown in each pot at equal distance. The pots of each treatment were arranged randomly, with three repeats at ambient light and temperature in a greenhouse. Tap water was used for irrigation. After germination, uniform plant population was maintained by thinning up to one plants pot^{-1}. The drought stress was applied by withdrawing water after 60 days of planting (flowering stage). The irrigation was first reduced and then completely stopped for a period of one week.

Plant Growth Measurements

Data of plant growth parameters including plant height, shoot biomass and root biomass were recorded. Plant height was measured before harvesting. Shoot and root biomass was recorded by uprooting the plant and drying at 72°C after harvesting.

Plant Ecophysiology Measurements

The plant physiological parameters were recorded at midday (between 11:00 and 13:30) of fully expanded leaves near the top of both irrigated and drought-stressed plants. Photosynthetic pigments of 3rd leaf from top were measured using a portable gas exchange system (Li-Cor 6400, Lincoln, NE, USA). During measurements, the leaves

were illuminated with lamp light (1500 µmol m^{-2} s^{-1} and maintained at a relative air humidity of 20±2% and a leaf temperature 25±2°C. Chlorophyll fluorescence was measured of irrigated and drought-stressed plants using a portable PEA handy (Hansatech Instruments Ltd., England). Leaves were dark adapted for 30 min before measurement and the maximum photochemical efficiency of PSII (Fv/Fm) was calculated from chlorophyll fluorescence data.

The leaf chlorophyll content was determined by using Chlorophyll Meter (SPAD 502 Plus). Each leaf sample was measured at least six different areas of each treatment with three replicates.

Electrolyte Leakage and Relative Water Content

Electrolyte leakage (EL) was measured following the protocol Jambunathan (2010), and relative water contents were determined following the equations described by Mayak et al. (2004).

$$EL\ (\%) = EC1/EC2 \times 100 \quad (1)$$

$$RWC\ (\%) = (\text{fresh weight - dry weight})/\text{fully turgid weight - dry weight} \times 100 \quad (2)$$

Persistence of *B. phytofirmans* PsJN in the Rhizosphere, Root and Shoot Interior

For the isolation of bacteria, 5 g rhizosphere soil and 3 g of surface-sterilized root/shoot materials was homogenized in 15 ml of 0.85% (w/v) NaCl solution. The homogenized material was shaken with pulsifier for 45 seconds at room temperature. After the settlement of materials, serial dilutions up to 10^{-4} were plated onto selective LB medium containing spectinomycin (100 µg ml^{-1}), 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (XGlcA) (100 µg ml^{-1}), and isopropyl-β-D-galactopyranoside (IPTG) (100 µg ml^{-1}). The plates were incubated at 28±2°C for 48 hours and then transferred to 4°C for three days. Blue colonies were counted on each plate. Thirty blue colonies of each treatment were randomly picked and the identity of isolates with the inoculant strain was confirmed by restriction fragment length polymorphism (RFLP) analysis of the 16S-23S rRNA intergenic spacer region (IGS) (Afzal et al., 2012).

Microscopy of Endophytic Colonization in Plant Tissues

Fresh plant organs (roots and leaves) were removed from inoculated and non-inoculated plant. Samples were then prepared for microscopy analysis as described by Compant et al. (2005), with some modifications. Briefly, plant samples were dipped in staining solutions containing IPTG (100 µg ml^{-1}) at 37°C for 48 hours. The samples were de-stained with 70% (v/v) ethanol solution to stop the reaction. Leaves and stem sections of plant were cut with a microtome (LeicaVT1000S; Leica, Nussloch, Germany), collected on glass slides, examined with an inverted Microscope (Axiovert 200 Carl Zeiss, Germany), and photographed.

Statistical Analysis

Data of plant growth parameters and bacterial densities were subjected to analysis of variance (ANOVA) using SPSS software package version 19 (IBM SPSS Statistics 19, USA). The treatment means were compared by Duncan's multiple range test at 5% probability. The means and standard errors were calculated using Microsoft Excel 2010.

RESULTS

Inoculation of maize seeds with *B. phytofirmans* PsJN increased plant height of both cultivars ranging from 14-24% as compared to control under irrigated and drought stress conditions (Table 1). Maximum increase was observed by inoculation (24%) in maize 'Mazurka' as compared to the respective control under drought stress.

Inoculation increased chlorophyll content significantly as compared to control. Up to 21% increase was observed by PsJN inoculation in 'Mazurka' under stress conditions. The poorest response to bacterial inoculation was observed in 'Kaleo' under normal

culture conditions (Table 1).

Likewise, inoculation increased the chemical efficiency of PSII as compared to control. Significant increase in PSII efficiency was observed in 'Kaleo' under drought stress (Table 1). Inoculation with strain PsJN increased photosynthesis activity of plants with the increase ranging from 18-68% as compared to control under normal and stressed conditions (Table 1). The maximum increase in photosynthesis (38%) was recorded in 'Mazurka' under stressed conditions. The weakest response (18%) to bacterial inoculation was observed in 'Kaleo' under normal culture conditions (Table 1).

The data in Table 2 revealed that inoculation with strains PsJN increased relative water content (RWC), electrolyte leakage (EL) and plant biomass as compared to the controls. Maximum RWC was observed in 'Kaleo' under normal conditions. However, inoculation with strain PsJN resulted in the highest increase in RWC (29%) as compared to control in 'Mazurka' under stressed conditions. The lowest increase in RWC was recorded in 'Kaleo' under normal conditions.

Bacterial inoculation decreased EL in both cultivars (Table 2). The strongest decrease was observed in 'Kaleo' under normal and stressed conditions.

Inoculation with strain PsJN significantly increased plant biomass (23-61%) of both maize cultivars over respective control under normal and stressed conditions. The strongest response to inoculation was observed in 'Mazurka' i.e., 43 and 61% increased biomass, respectively as compared to control under both conditions. The poorest response was observed in 'Kaleo' i.e., 23% increase in shoot biomass over control under normal conditions.

Similarly, PsJN inoculation increased root biomass of both cultivars i.e., 38-62% as compared to control under normal and stressed conditions (Table 2). 'Mazurka' grown under drought stress conditions showed strongest response (62%) to the bacterial inoculation, whereas plants of 'Kaleo' grown under normal water regime showed the lowest increase in root biomass as compared to the as compared to the corresponding control.

B. phytofirmans PsJN efficiently colonized rhizosphere, root, shoot and leaf interior of both maize cultivars, 'Mazurka' and 'Kaleo' (Figs. 1 and 2). However, an about ten times higher viable cell number (CFU/g dry weight) was recovered for the rhizosphere and roots as compared to shoot tissue (Fig. 1). In general, we recorded a higher viable cell number in plants of 'Mazurka' than in 'Kaleo'. In stressed plants of both cultivars the number of viable PsJN cells was remarkably lower than in plants treated with a normal water regime.

DISCUSSION

In changing climate, plants are constantly exposed to abiotic stress, such as drought, which is one of the most serious problems associated with plant growth and development affecting agricultural demands. The management of drought-affected soils is essential to meet the ever increasing food demands. Inoculation with plant growth promoting bacteria (PGPB) has been found effective under drought stress environment (Chanway and Holl, 1994) to increase productivity. Growth promotion by the PGPB may be attributed to multifarious mechanisms such as production of PGP hormones and other PGP activities (Glick, 1995). In the present investigation, plant growth-promoting endophytic bacterium was evaluated on the growth and photosynthesis of maize cultivars under drought stress conditions.

The inoculation of maize plants with the bacterium *B. phytofirmans* PsJN stimulated plant biomass production, physiology and vitality in both cultivars. From numerous reports it is evident that *B. phytofirmans* PsJN is a highly efficient plant beneficial bacterium promoting growth in a wide variety of plants (Compant et al., 2008), however, there is evidence for plant genotype specific differences in the intensity of the effects (Da et al., 2012; Trognitz et al., 2008). Interestingly, this can be seen also from the present data. Cultivar 'Mazurka' responded stronger to inoculation with strain PsJN than cultivar 'Kaleo' in both drought stress and normal water conditions, and this was more

pronounced in stressed plants. Nowak and colleagues assumed that plant genotype specific differences in the plant stimulating effects is due to differences in PsJN titers in highly and poorly responsive cultivars, which reach much higher levels in highly-responsive genotypes (Nowak et al., 2007). The data of not drought stressed plants indicate a correlation between stimulation and PsJN titers as we recorded a higher number of viable PsJN cells in 'Mazurka' than in 'Kaleo'. However, in drought stressed plants strain PsJN was more suppressed and the viable cell number dropped more drastically than in 'Kaleo', while in the latter cultivar the viability of strain PsJN seemed hardly affected. The number of viable PsJN cells in stressed plants of 'Mazurka' was far below that of 'Kaleo' but at the same time the relative increase in plant growth and vitality under drought was much higher.

By comparing the performance of the two maize cultivars under drought stress we saw that 'Kaleo' was less negatively affected than 'Mazurka'. Overall biomass production, photosynthesis and water content were higher and electrolyte leakage lower in plants of 'Kaleo', indicating that 'Kaleo' was more resistant towards drought stress in our experiment as compared to 'Mazurka'. In general, inoculation with *B. phytofirmans* PsJN significantly minimized the negative effects of drought on maize biomass production and physiology. Similar effects were observed when *B. phytofirmans* PsJN was tested for its ability to enhance chilling resistance in *Vitis vinifera* L. 'Chardonnay' (Ait Barka et al., 2006; Fernandez et al., 2012). The higher tolerance of PsJN colonized grapevine plantlets to chilling were related to alterations in photosynthesis and sugar metabolism. Recently, Theocharis et al. (2012) showed that cold stress-related gene transcripts and metabolites increased earlier and faster, and reached higher levels in PsJN-colonized 'Chardonnay' grapevine plantlets than in control plants.

In conclusion, *B. phytofirmans* PsJN efficiently colonized maize plants and stimulated plant growth in both cultivars tested. Our study clearly demonstrates that bacterial inoculants could be used to minimize the negative effects of drought stress on growth and photosynthesis of maize.

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Tables

Table 1. Effect of *Burkholderia phytofirmans* strain PsJN on plant height, chlorophyll content, chlorophyll fluorescence and photosynthesis of maize under drought stressed conditions.

	Mazurka		Kaleo		Mazurka		Kaleo	
	Plant height (cm)				Chlorophyll content (SPAD value)			
	N H_2O[†]	D H_2O[‡]	N H_2O	D H_2O	N H_2O	D H_2O	N H_2O	D H_2O
Control	182.53±3.50c	167.67±2.52d	179.98±3.82c	164.33±2.08d	39.03±1.53de	34.93±1.55f	40.60±2.31cd	36.90±1.31ef
PsJN	208.67±3.05a	207.33±3.52a	205.33±2.30ab	202.33±2.07b	45.80±1.51a	42.10±0.85bc	44.47±0.93ab	44.13±0.68ab
	Chlorophyll fluorescence (Fv/Fm)				Photosynthesis (μmoles CO_2 cm^{-2} s^{-1})			
Control	0.780±0.01b	0.739±0.01c	0.796±0.02ab	0.754±0.01c	18.81±2.45c	10.73±1.12d	22.13±2.65b	17.44±2.26c
PsJN	0.823±0.01a	0.802±0.02ab	0.817±0.01a	0.811±0.01 a	26.11±1.48a	18.03±1.56c	25.99±1.56a	23.54±1.19ab

Means sharing similar letter(s) in a column for each parameter do not differ significantly at P=0.05.
Data are average of three replicates ± Standard Deviation (SD).
[†] Normal irrigation.
[‡] Reduced water application.

Table 2. Effect of *Burkholderia phytofirmans* strain PsJN on relative water content, electrolyte leakage, shoot dry matter and root dry matter on maize under drought stressed conditions.

	Mazurka		Kaleo		Mazurka		Kaleo	
	Relative water content (%)				Electrolyte leakage (%)			
	N H_2O[†]	D H_2O[‡]	N H_2O	D H_2O	N H_2O	D H_2O	N H_2O	D H_2O
Control	52.17±2.67d	44.50±1.62e	64.96±2.06b	58.29±1.86c	8.06±1.74bc	12.06±1.33a	7.76±1.65bc	10.15±1.14ab
PsJN	58.31±1.12c	57.31±2.59c	70.88±1.45a	67.88±2.32ab	6.29±1.21d	7.95±0.55bc	5.79±0.89d	7.02±1.47cd
	Shoot dry matter (g)				Root dry matter (g)			
Control	21.13±2.01de	18.40±1.77g	57.63±1.82de	22.07±1.50f	2.48±0.09b	1.53±0.11c	2.46±0.23b	1.63±0.07c
PsJN	37.38±1.20a	29.57±1.66cd	33.98±1.47b	32.34±1.79bc	3.50±0.18a	2.47±0.13b	3.39±0.08a	2.56±0.08b

Means sharing similar letter(s) in a column for each parameter do not differ significantly at P=0.05.
Data are average of three replicates ± Standard Deviation (SD).
[†] Normal irrigation.
[‡] Reduced water application.

Figures

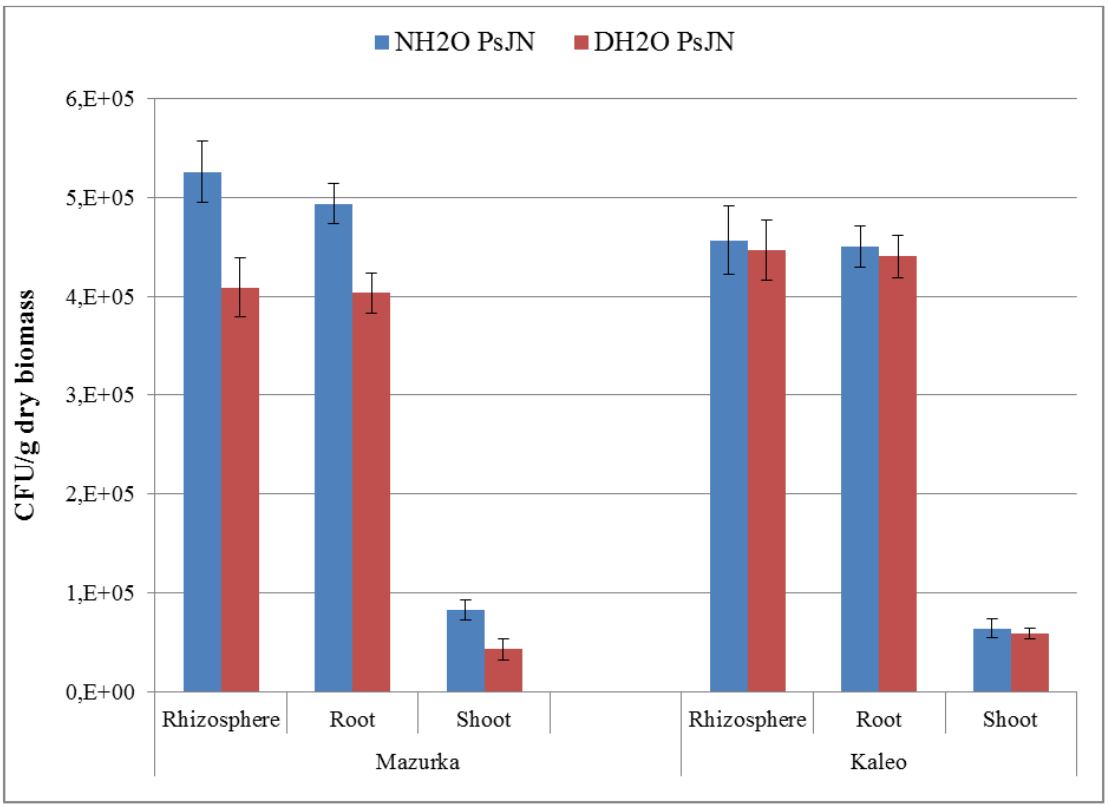

Fig. 1. Persistance of *Burkholderia phytofirmans* strain PsJN in the rhizosphere, root interior and shoot interior of two maize cultivars under normal and water stressed conditions.

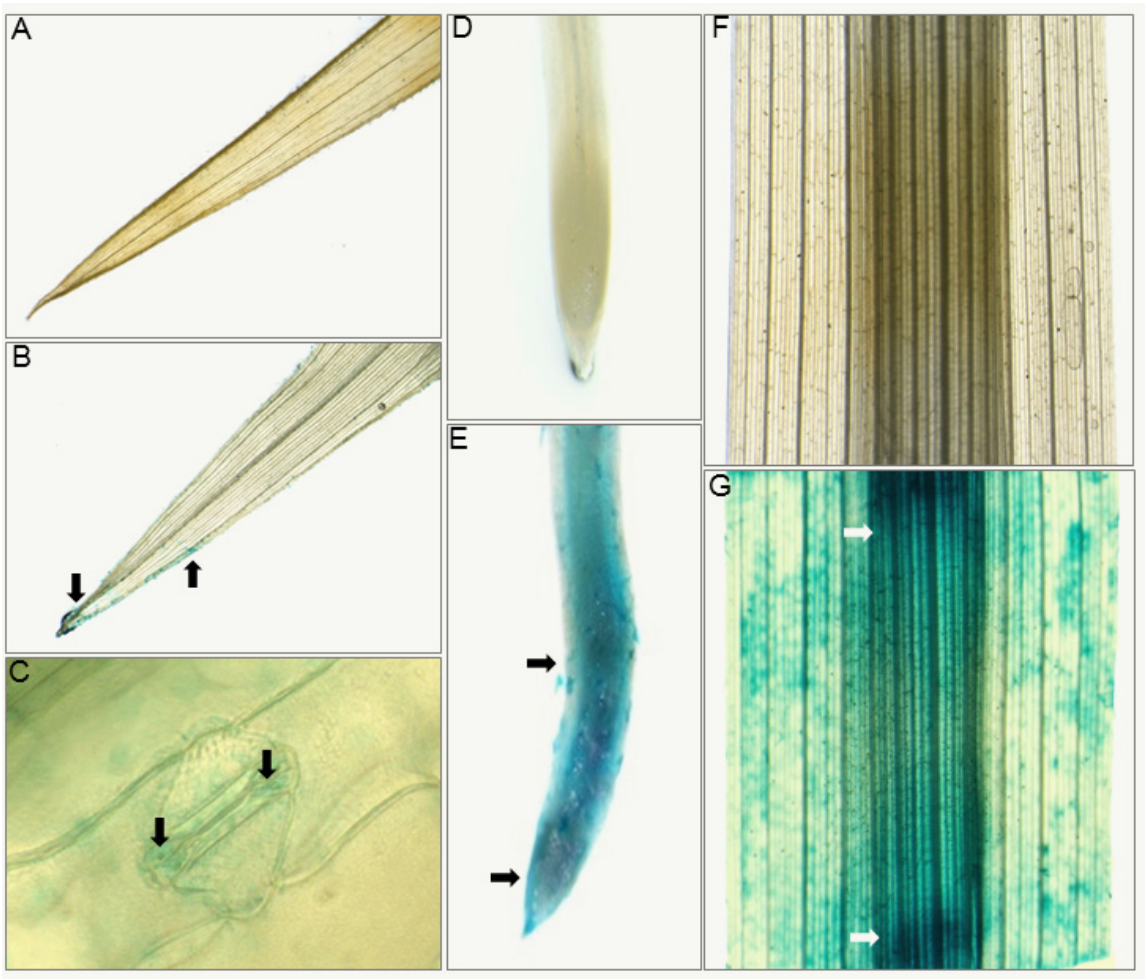

Fig. 2. Photographs of the fourth leaf internal tissue of PsJN inoculated *Zea mays* L. plants. (A, B, F and G) Photographed (binocular microscope; Olympus, Japan) of the fourth leaf (tip and middle) and root of uninoculated control (A, D and F) or inoculated with PsJN: gusA10 (B, E and G), showing the blue color in veins due to gusA-marked cells (arrowheads). Inverse microscope (Axiovert 200M; Zeiss, Hallerbergmos, Germany) image of the leaf stomata of PsJN gusA inoculated plant, showing bacteria in the stomata and guard cell.