

## RESEARCH ARTICLE

# Characterization of *Pseudomonas savastanoi* pv. *Glycinea*, the cause of bacterial blight in soybean

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## ABSTRACT

*Pseudomonas savastanoi* pv. *glycinea* isolated from tan coloured spots on the cotyledons of soybean was gram negative, short rod, 0.6-0.8 × 1.2-30 μ, non spore former and motile by a single polar flagellum. King's B medium was best supporting for the growth of bacterium. Growth of the bacterium was maximum at temperature 25°C and pH 6.8. The pathogen was unable to liquify gelatin and produced acid from glucose and sucrose but not from starch and was not able to produce gas from any of the carbon sources tested and also failed to hydrolyse starch and H<sub>2</sub>S production. Growth of the bacterial isolate was maximum in King's B medium supplemented with arabinose, ammonium sulphate and tryptophan.

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## INTRODUCTION

*Pseudomonas savastanoi* pv. *glycinea* causing bacterial blight of soybean is responsible for reducing the economic yield of the crop world wide where soybean is extensively grown (Kendrick and Gardner, 1921 and Dowson, 1957). Currently blight is most common bacterial disease of soybean especially in cool and wet weather. In present investigation the bacterium was studied for morphological, cultural and physiological characteristics.

## MATERIALS AND METHODS

Morphological characters of the bacterium were studied using different staining procedures and motility test. Gram's staining was used for diagnostic identification of bacterium. Colony of bacteria growing on agar medium was suspended in a drop of water on a slide and air dried for spore determination. Slide was flooded with 5.0 per cent (w/v) aqueous malachite green, allowed reacting for 30-40 seconds and then the slide was heated until it steams and counter stained by flooding with 0.5 per cent (w/v) aqueous safranin for 15 seconds. The slide was rinsed thoroughly with water,

blot dried and the bacterial cells were observed under oil immersion. For observing the presence of flagella, 12 to 18 h young culture grown on nutrient agar in slants was taken and distilled water was poured slowly, without disturbing bacterial growth and the tubes were kept standing for 2-3 minutes to allow the motile cells to come into suspension. A pipette was dipped into the bacterial suspension and placing a drop of suspension on slide, tilted and air dried. Smear was marked on the opposite side and covered with mordant solution (A+B) (mordant solution A= Mercuric chloride C.P (saturated aqueous solution) 2.0 ml, tannic acid 2.0 ml, aluminum potassium sulphate 5.0 ml, B= basic fuchsin (saturated aqueous solution) 0.04 ml and allowed it to act for 8-10 minutes. After washing the slides with distilled water smear covered with Ziehl-Neelson's Carbol fuchsin stain on the slide, allowed to stain for 5 minutes, subsequently washed in tap water, air dried and examined under oil emersion.

Eight different media (M-71, tryptic soy agar (TSA), D<sub>4</sub>, and SVCA, yeast extract dextrose calcium carbonate (YDC), King's B medium (KB), nutrient glucose agar (NGA) and TTCC medium were tested to identify the colony characters of the bacterium. Bacterial suspension (0.5 ml) of 24 h old culture

0.2 M K <sub>2</sub> HPO <sub>4</sub> (ml)	0.1 M citric acid (ml)	KB broth (ml)	Total volume (ml)	pH
0.9	1.1	8	10	4.4
1.1	0.9	8	10	5.2
1.3	0.7	8	10	6.0
1.5	0.5	8	10	6.8
1.9	0.1	8	10	7.6
0.2 M NaOH (ml)	0.2M boric acid (ml)	KB broth (ml)	Total volume (ml)	pH
0.31	1.7	8	10	8.4

was added to each 250 ml flask having 50 ml of sterilized broth and incubated at 27°C. Observations were recorded after 12, 24, 30, 36, 48, 60, 72, 84 and 96 h of incubation in terms of optical density using thermo spectrophotometer at 620 m $\mu$ .

Effect of temperature on the growth of *Pseudomonas savastanoi* pv. *glycinea* was studied using King's B Broth medium. Twenty five ml of the media was dispensed in 100 ml flasks, sterilized and inoculated with 24 h old bacterial culture. After 2 days of incubation at 15, 20, 25, 28, 30 and 35°C temperatures, observations were recorded in terms of optical density, Different pH levels (4.4, 5.2, 6.0, 6.8, 7.6 and 8.4) were also tested for growth of the bacterium. To get the medium of required pH value, following solutions were prepared and mixed in the following proportion.

The medium was inoculated with 24 h old actively growing bacterial culture under aseptic conditions and observations in terms of optical density were recorded after 48 h of incubation at 25 $\pm$ 1°C.

Gelatin liquefaction using gelatin medium as basal medium was carried out to take observations on gel liquefaction. Five ml of basal medium after sterilization was taken in each test tube maintaining 3 replications and inoculated by stabbing with inoculating needle using 24 h old culture. These inoculated tubes along with check were incubated at 25 $\pm$ 1°C and observations were recorded at 7 day intervals up to 30 days. The test tubes were kept into refrigerator for further observation on gel liquefaction. To know the character of producing hydrogen sulphide gas, peptone water medium was used as basal medium. Whatman No. 1 filter paper strips (10x50 mm) were cut and dipped into saturated lead acetate solution. Strips were dried, sterilized and again dried in oven at 60°C. Five ml. medium was dispensed in test tube inoculated with one loop bacterial suspension and test strips were inserted between the cotton plug and inner wall of culture tube, not touching the broth and 14 days after incubation at 25 $\pm$ 1°C observations were recorded.

Starch broth medium was used as basal medium for starch hydrolysis. This was added with agar, sterilized and poured into sterilized Petri plates. On solidification, medium was spot inoculated at 25 $\pm$ 1°C. For hydrolysis, the plates were taken out from the incubator after 3, 4, 7 and 14 day intervals and the

surface was flooded with lugolo's iodine and allowed to act for few minutes. For detection of acid and gas production by bacteria, utilizing different carbon sources, Ayers Rupp and Johnson's medium was used as a basal medium added with different carbon sources viz., glucose, sucrose and starch (sterilized separately). Carbon sources @ 1.0 per cent were then added aseptically in the medium of each carbon source, separately and the slants were prepared, inoculated with 48 h old culture and incubated at 25 $\pm$ 1°C. Observations were recorded at 2 day intervals for 7 days. Ten different carbon sources (glucose, galactose, xylose, arabinose, maltose, lactose, sucrose, glycerol, dextrin and mannitol) @ 0.1 per cent were added to King's B medium to evaluate the growth of bacterium. Twenty five ml of medium was poured in each 100 ml flasks, inoculated and incubated at 25 $\pm$ 1°C. Observations were recorded after 24, 48, 72, and 96 h of incubation in terms of optical density. To know the utilization of inorganic and organic nitrogen sources by the bacterium (ammonium dihydrogen orthophosphate, aluminium ammonium sulphate, diammonium hydrogen orthophosphate, ammonium chloride and ammonium nitrate added to King's B medium as a substitute of inorganic nitrogen source @ 0.1 per cent and 12 amino acids ( alanine, methionine, aspartic acid, proline, leucine, tryptophan, arginine, serine, glycine, glutamic acid, phenylalanine and cystine) @ 10 mg/l were added to King's B medium as organic nitrogen source. The flasks containing sterilized media were inoculated with 24 h old bacterial culture and incubated at 25 $\pm$ 1°C. Observations in terms of optical density were recorded after 24, 48, 72 and 96 h of incubation.

## RESULTS AND DISCUSSION

The experimental findings obtained from the present study have been discussed in following heads:

### Morphological details :

After gram's staining, the bacterial cells stained to pink in colour with dark coloured margin exhibiting the pathogen as gram negative rods appear singly or in pairs, occasionally in chains of 2-4 measuring 0.6-0.8 x 1.2-3.0  $\mu$  in size, motile by polar flagellum and the pathogen was non spore forming. Rod

shaped bacterium with rounded ends appearing singly or in pair or in chains was also reported by walf (1920).

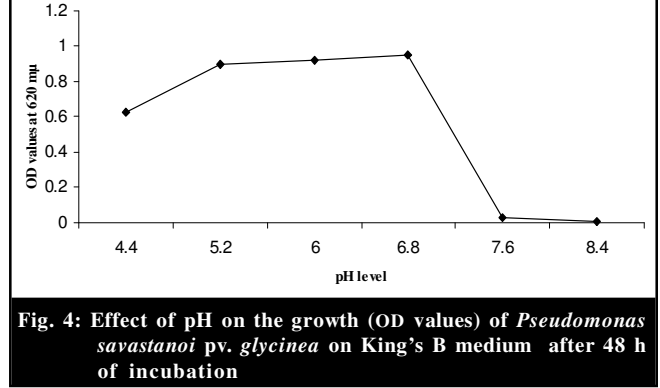
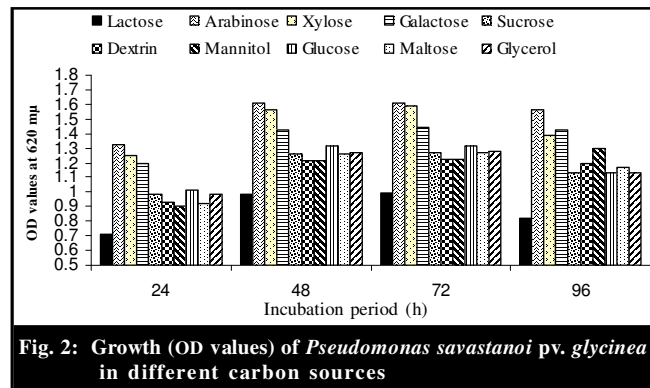
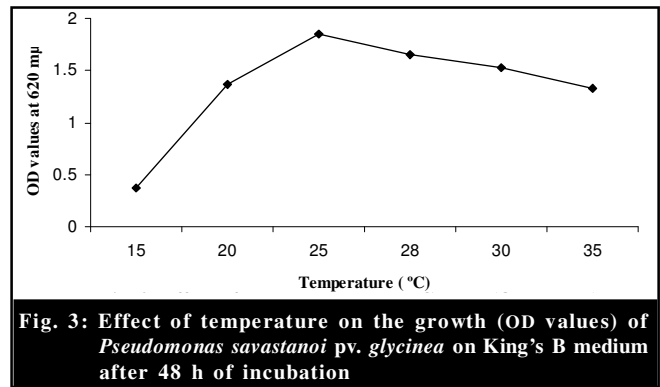
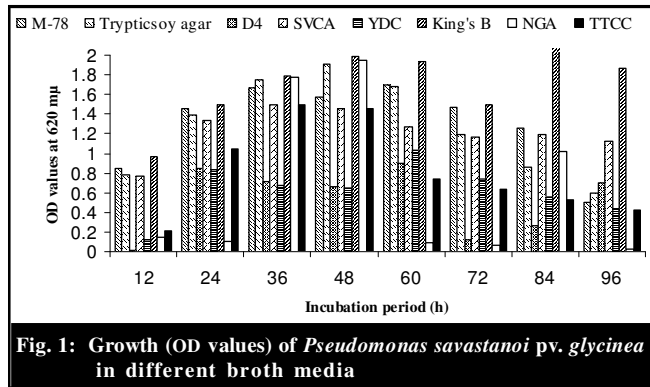
**Cultural characters :**

The data on growth of the bacterium on different test media revealed that after 12 h of incubation, the maximum growth was in KB (0.971) followed by M-71 (0.843) while minimum growth was recorded in D4 (0.012) followed by YDC medium (0.120). After 24 h of incubation, growth of bacterium was maximum in KB (1.503) followed by M-71 medium (1.454). After 36 h of incubation maximum growth was in KB (1.792) followed by TSA (1.743) while the growth was minimum in YDC (0.671) medium. After 48 h of incubation, KB medium supported the maximum growth (1.983) while it was minimum in YDC medium (0.654) and similar results were obtained after 60, 72, 84 and 96 h of incubation where KB medium supported maximum growth 1.937, 1.997, 2.063 and 1.862, respectively, while minimum growth was recorded in TTCC (0.746), NGA

(0.066), TTCC (0.528) and NGA medium (0.022) respectively (Fig.1). King *et al.* (1954) reported that for successful culturing of *P. glycinea*, King’s B medium suited best.

**Growth at different temperature and pH levels :**

Growth of the bacterium was statistically higher at 20, 25, 28, 30 and 35°C than at 15°C (Fig.3). Maximum growth (1.848) was obtained at 25°C followed by 28°C (1.647) on King’s B medium after 48 h of incubation while checking the growth of bacterium in different pH level it was observed that at pH 6.8 on King’s B medium at 25±1°C maximum growth (0.954) was recorded followed by pH 6.0 (0.919) (Fig.4). The present findings are in accordance with Buchanan and Buchanan (1951); Sinclair and Backman (1989), who reported that the bacterium grew well at 25-30±1°C and pH 6.8-7.0 suited best for the growth of *Pseudomonas savastanoi* pv. *glycinea* (Burkholder, 1957)



**Table1: Mode of utilization of carbon sources for acid and gas production by *Pseudomonas savastanoi* pv. *glycinea***

Incubation period (days)	Acid production			Gas production		
2	+	-	-	-	-	-
4	++	+	-	-	-	-
7	+++	++	-	-	-	-

(+) Positive reaction for acid or gas production, (-) Negative reaction for acid or gas production

### Physiological studies :

The result of experiment conducted for confirmation of gelatin liquefaction revealed that gelatin was not liquefied by the bacterium even after 28<sup>th</sup> day of observation when the tubes were kept in refrigerator. While going for the study on production of H<sub>2</sub>S gas, no blackening of the lead acetate filter strip was noticed after 14<sup>th</sup> day of incubation showing the inability of the test bacterium to produce H<sub>2</sub>S gas. For starch hydrolysis test observations were recorded for colour change and no change was recorded in contrast to blue colour in the area surrounding the spot (where bacterium was inoculated) which shows negative reaction about starch hydrolysis by the test bacterium. Burkholder (1957) also observed that *Pseudomonas glycinea* do not hydrolyses the starch.

### Utilization and mode of utilization of carbon sources :

Graph showing the growth of *Pseudomonas savastanoi* pv. *glycinea* supported by different carbon sources revealed that maximum growth was recorded in arabinose (1.323) followed by xylose (1.255) and minimum in lactose (0.713) after 24 h of incubation and similar observations were recorded after 48 h. After 72 h of incubation there was non significant higher growth obtained from arabinose (1.611) and xylose (1.585) but differed better than other carbon sources whereas, after 96 h of incubation maximum growth was recorded in arabinose (1.561) followed by galactose (1.421) with minimum in lactose (0.817) (Fig.2) The variability in the utilization of different carbon sources by different isolates of *Pseudomonas savastanoi* pv. *glycinea* was also reported by Mishagi and Grogan (1969). In the present study all the carbon sources were utilized by the bacterium but in variable amount. Mode of utilization of carbon sources observed on the basis of colour change from reddish violet to yellow for acid production and cracks in media for gas utilization revealed that the bacterium utilized glucose and sucrose for acid production but starch was not utilized for acid production, however, gas production was not observed in any of the carbon sources (Table 1). Burkholder (1957) also observed similar results that acid was produced from glucose and sucrose but not from starch and gas were not produced by any of the carbon sources.

### Utilization of inorganic and organic nitrogen sources :

All the inorganic and organic nitrogen sources supported the growth of the bacterium. Among the inorganic nitrogen sources (Fig. 5) maximum growth (0.326) was recorded in ammonium sulphate followed by ammonium hydrogen orthophosphate (0.259) and minimum growth (0.085) in ammonium chloride after 24 h of incubation. Similar pattern was also followed up to 96 h of incubation. The results of this study are in agreement with those of Misaghe and Grogan (1969) who observed that *Pseudomonas savastanoi* could utilize different nitrogen sources viz., ammonium sulphate,

ammonium nitrate, ammonium phosphate and potassium nitrate for growth. The bacterium responds well in all organic nitrogen sources at different observation levels (Fig. 6). Significantly higher growth was recorded in tryptophan followed by glycine as compared to other organic nitrogen sources and minimum growth was recorded in leucine after 24, 48, 72 and 96 h of incubation. Missaghi and Grogan (1989) reported that *Pseudomonas savastanoi* had a preference for L-leucine, D-gutamate, alanine and serine *in-vitro* conditions. It has been reported that strains of *Pseudomonas* preferred tryptophan, alanine, arginine and serine. This may be due to different sources of acquiring the nitrogenous compounds under the present investigation and the isolate is different.

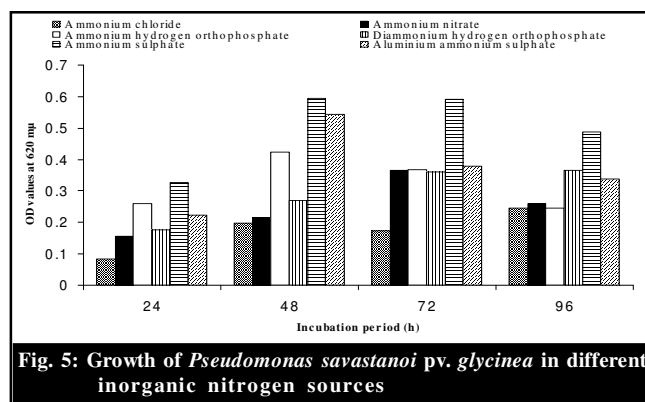


Fig. 5: Growth of *Pseudomonas savastanoi* pv. *glycinea* in different inorganic nitrogen sources

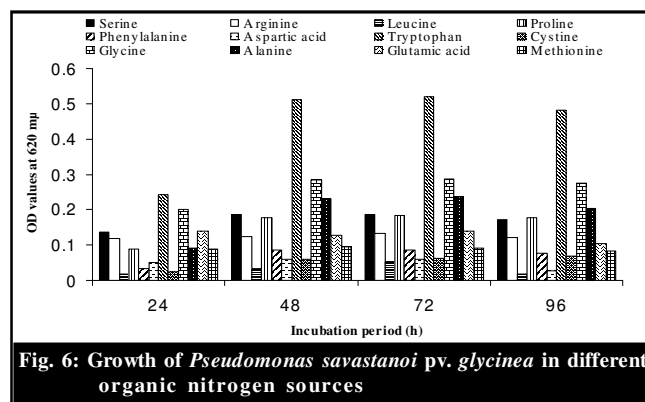


Fig. 6: Growth of *Pseudomonas savastanoi* pv. *glycinea* in different organic nitrogen sources

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