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Development of a Carrier Based Formulation of Sulphur Oxidizing Bacterium for Enhancing the Productivity of Crops Requiring Sulphur Nutrition

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ABSTRACT

A total of nine sulphur oxidizing bacterial isolates were obtained from four different sources using Starkey broth, NCL broth and Sodium thiosulphate broth. The isolates were characterized and screened based on pH reduction and sulphate production to get the efficient isolate. The isolate BGS2 reduced the pH upto 3.5 and produced 79.2 mg per 100 ml broth of sulphate and it is formulated with lignite as carrier material. Lignite based formulation recorded the highest population of $1X10^{10}$ CFU per g of formulation on 90^{th} day. Moreover, lignite based formulation stored at different temperatures showed that packets stored under refrigerated condition as well as in mud pot covered with wet cloth recorded the highest population when compared to high temperature and room temperature incubation.

KEY WORDS: BGS2, pH reduction, sulphate production, powder formulation, different temperature.

INTRODUCTION

Sulphur is one of the basic building blocks in Microorganisms, plants and animals and hence it is considered to be vital for life. In plants, it is considered as the fourth essential nutrient next to N, P and K. However, its wide spread deficiency in soils and consequent losses on crop productivity have been reported during last three decades due to the continuous use of sulphur free fertilizers and intensive cultivation with high yielding varieties [1]. So, the crop plants have become increasingly dependent on the soil to supply the sulphur that they need for the synthesis of proteins and a number of essential vitamins and co- factors [2]. Plants uptake sulphur in the form of sulphate.

Chemically processed fertilizers are largely water soluble and contain high and immediately available nutrient concentrations. But, the process increases fertilizer cost and makes it unaffordable for a large proportion of the typically small, family-based farmers involved in food production. This induced researches on alternative fertilizers. Moreover, the immediate utilization of such fertilizers are restricted due to the low solubility and work to establish the agronomic efficiency and economic use of phosphate rocks is not yet conclusive. So, an alternative has been the use of microorganisms with ability to promote nutrients.

The soil microbial biomass is the key driving force behind all sulphur transformations. The biomass acts as both a source and sink for inorganic sulphate. They make available sulphate from element or any reduced forms of sulphur, through oxidation process in the soil [3]. Uses of sulphur oxidizers enhance the natural oxidation and speed up the production of sulphates.

Bio inoculants are most often made by incorporation of the microbial inoculum into solid carrier, which provides a convenient base for packing and facilitates application and use of the product [4]. Several workers have proved that peat based inoculant is still considered to be the most dependable and also accepted worldwide [5]. Besides low grade quality and limited availability, high charges involved in transporting it from the source, prohibit the extensive use of peat for commercial manufacture of legume in India [6].

Lignite is now regarded as a standard carrier and also adopted for commercial production of legume inoculants, as well as for other nitrogen-fixing cultures in India [7]. Carrier is a medium matrix on which the inoculated microorganisms grow to a reasonably higher population for an initial period and there after decline. The nature of the carrier often determines the subsequent performance of the inoculant. Early cultures were essentially the agar cultures of today but they changed to carrier-based culture to overcome short shelf life of agar and liquid cultures [8]. Lignite based inoculants are widely accepted and used for seed treatment of various crops [9].

In this study we made an attempt to isolate sulphur oxidizing bacteria from different ecological niches of India and developed powder formulation for seed and soil inoculation.

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MATERIALS AND METHODS

Isolation and characterization

For isolation of heterotrophic sulphur oxidizing bacteria, Starkey broth, NCL broth and thiosulphate medium were employed by enriching the Starkey broth, NCL broth with elemental sulphur and thiosulphate medium with sodium thiosulphate and the above medium is modified by the addition of 5g yeast extract and glucose. The initial pH of all the growth media was adjusted to 8.0 using 0.1N KOH (Potassium hydroxide). Bromocresol purple served as the indicator. The broth was sterilized for three consecutive days. One gram or one ml of the sample was added to 10 ml of the broth contained in tubes, under aseptic conditions. The tubes were incubated at 37°C for 3 days. The isolates obtained were purified by transferring to fresh broth thrice at fortnightly intervals. The isolates were then streaked on thiosulphate agar medium and individual colonies were picked and used for further studies.

Phenotypic characterization of Sulphur Oxidizing Bacterial isolates

Physiological and biochemical characters of sulphur oxidizing bacterial isolates were examined according to Bergey's manual of determinative bacteriology [10]. All cells used for characterization studies were grown in thiosulphate agar medium. Gram staining was performed by the Hucker method [11]. The screened isolates were studied for their utilization of sulphur sources. The screened isolates were inoculated in sterilized elemental sulphur broth as well as Thiosulphate broth (initial pH 8.0) and incubated at 37°C for 3 days. The growth was assessed by pH reduction and microscopic observation.

Thiosulphate agar medium was prepared with pH adjusted to 8.0. The screened isolates were plated in sterile Petri dishes by pour plate method and the plates incubated at 37°C for 3 days. Colony characters were observed after the incubation period. Thiosulphate broth with initial pH of 8.0 was prepared with and without glucose. Screened isolates were inoculated and incubated at 37°C for 3 days. Based on the pH reduction in the broth with and without glucose, isolates were classified as chemoheterotrophs. Growth of sulphur oxidizing bacteria at different temperatures (29°Cto 45 °C) was tested by incubating them in incubator at desired temperature.

Screening of isolates

The isolates were screened based on pH reduction and sulphate production to obtain an efficient isolate.

pH reduction test

The obtained isolates were inoculated in the growth media with initial pH adjusted to 8.0. After 3 days of incubation the final pH of the growth media was measured using pH meter. The isolates were screened based on their efficacy to reduce the pH from 8.0 to 4.5. The screened isolates were used for further studies.

Assay for Sulphate production

Extracellular sulfate productions by bacterial isolates were determined in thiosulphate broth with sodium thiosulphate 5 % (w/v). Three days old cultures were used for estimation of sulphate production. Cultures were centrifuged at 10000 rpm for 15 min. A quantity of 0.1ml of supernatant was taken in a 25ml volumetric flask and volume was made up to 25 ml with distilled water. 1.0 $Bacl_2$ powder was added to the samples and shaken well. It was kept for 1min. The reading was taken in Cary with UV optical spectrophotometer at 340nm wavelength (Varian 8510162500, Software connected imported from Varian Australia Pvt.Ltd., Australia). Simultaneously standards were run with known quantity of K_2SO_4 [12].

Studies on the development of formulation of sulphur oxidizing bacteria
Standardization of pH in formulation development

Lignite and rock phosphate were the two major ingredients used for the preparation of formulation. Lignite should be pulverized, sieved to get powdered texture and sterilized the lignite and rock phosphate were combined in different proportions and best combination was selected based on pH value. Twenty grams of carrier material containing lignite and rock phosphate from each treatment was weighed and 40ml of distilled water was added. The sample was then vortexed for 5 minutes to get uniform mixing of carrier with the distilled water. The vortexed sample was allowed to settle for 30 min and then pH of each treatment was determined using Elico pH meter. The combination that recorded near neutral pH value was taken for further studies.

Standardization of sodium thiosulphate quantity for formulation development

To fix the right level of sodium thiosulphate for the development of formulation, sodium thiosulphate was added at different proportions at the rate of 2.5% and 5% and control with lignite (50g) and Rock phosphate (50g). Calcium carbonate was added up to the level of bringing the carrier pH to 7.0. A quantity of 50 ml of 48 hours old culture was mixed with 100g carrier. The formulation was kept for storage at room temperature. The initial population was 5 X 10^{11} cfu/ml. Based on survival of sulphur oxidizing bacteria, the best combination was used for further studies.

Enumeration of sulphur oxidizing bacterium in the carrier

The shelf life of the bacterium in the carrier was evaluated by enumeration of bacteria. Ten grams of inoculum was taken and aseptically transferred to 90 ml of sterile distilled water and shaken well. The enumeration was carried out using pour plate technique on modified sodium thiosulphate agar medium. Plates were kept for incubation and expressed as colony forming units (CFU) per gram of bacterial carrier.

Influence of different incubation temperature on the formulation during storage (7)

For developing carrier based powder formulation, lignite and rock phosphate were mixed in the ratio of 1:1. Five percent of sodium thiosulphate and Calcium carbonate was added upto the level of bringing the carrier pH to 7.0. A quantity of 50 ml of 48 hours old culture were also mixed. The final proportion of carrier material (lignite and rock phosphate) with culture was 1:1:1. The initial population is 5 X 10^{11} cfu/ml. The formulation was well packed in polythene bags and kept for storage at different temperature such as high temperature (42° C), room temperature, refrigerated condition (4° C) and farmers practice (keeping in mud pot covered with moist cloth).

Evaluation of shelf life of the isolate in carrier formulation by enumerating the BGS2 in the carrier

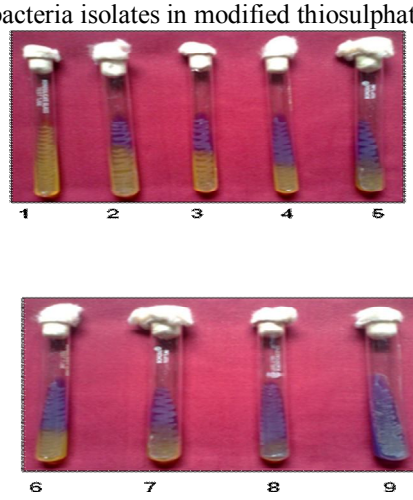
The shelf life of the bacterium in the carrier was evaluated by enumeration. Ten gram of carrier was taken and aseptically transferred to 100 ml of sterile distilled water and shaken well. The enumeration was carried out using pour plate technique on sodium thiosulphate agar. Plates were kept for incubation and the growth was expressed as colony forming units (CFU) per gram of carrier.

RESULTS AND DISCUSSION

Isolation

A total of nine sulphur oxidizing bacterial isolates were obtained from four different sources (Plate 1). Anandham *et al.,* [13] isolated nine chemolithoautotrophic and twelve chemolithoheterotrophic sulphur oxidizing bacteria using enrichment technique in modified Starkey's medium from different ecological niches. Twenty eight sulphur oxidizing bacterial isolates were isolated from different samples such as paddy rhizosphere, pulse rhizosphere, sewage, biogas slurry, tannery effluent and mine soil [14].

Plate 1. Purified Sulphur oxidizing bacteria isolates in modified thiosulphate slants



1 - BGS2 2 - TRY2 3- SWG2 4- TRY1
5 - TRY3 6- BGS1 7 – BGS3 8- SWG1
9- ALR1

Characterization of isolates

Based on the morphology, colony characters, Gram reaction and nutritional requirement, the selected isolates were characterized and the results are presented in Table 1. All the isolates were found to be short rods and Gram positive and the heterotrophic colonies were smooth, round, straw yellow colored (Plate 2). Similar morphological characteristics *viz.,* smooth, circular and short rods were reported Graff and Stubner, [15]. They were able to reduce the pH from 8.0 (Plate 3) and utilize thiosulphate as sulphur source and glucose as carbon source. Sulphur oxidizing bacteria obtain energy by oxidation of thiosulphate and elemental sulphur [16]. Isolates were positive for catalase activity. All the isolates were positively influenced by biotin, which was concurrent with the findings of Kelly and Harrison, [17]. All the isolates had temperature tolerance up to 37^0C.

Table 1. Morphological, biochemical and physiological characteristics of SOB isolates

SOB Isolates	SWG1	SWG2	TRY1	TRY2	TRY3	BGS1	BGS2	BGS3	ALR1
Shape	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Gram staining	+	+	+	+	+	+	+	+	+
Utilization of Elemental Sulphur	-	-	-	-	-	-	-	-	-
Utilization of Thiosulphate	+	+	+	+	+	+	+	+	+
Utilization of Glucose	+	+	+	+	+	+	+	+	+
Utilization of Yeast Extract	+	+	+	+	+	+	+	+	+
Influence of biotin	+	+	+	+	+	+	+	+	+
Catalase test	+	+	+	+	+	+	+	+	+
Temperature tolerance 29°C	+	+	+	+	+	+	+	+	+
35°C	+	+	+	+	+	+	+	+	+
37°C	++	++	++	++	++	++	++	++	++
40°C	-	-	-	+	-	-	+	+	+
45°C	-	-	-	+	-	-	-	-	-

Plate 2. Growth of sulphur oxidizing bacteria in modified thiosulphate agar medium

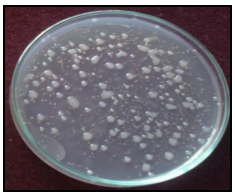


Plate 3. Growth of sulphur oxidizing bacteria in modified thiosulphate broth

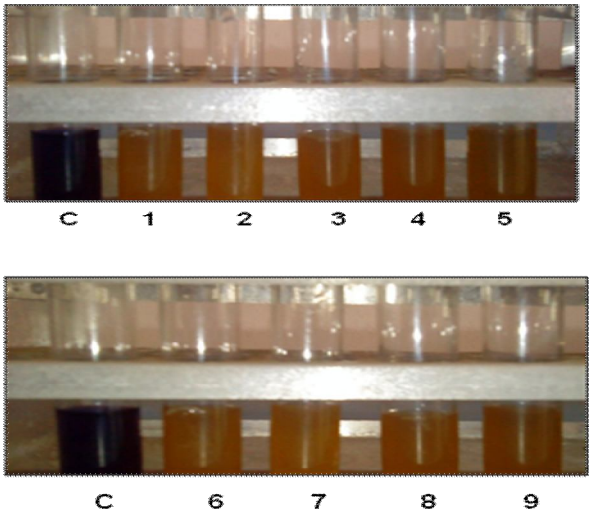
Screening of sulphur oxidizing bacterial isolates
Screening by pH reduction test

The nine isolates were screened based on the pH reduction. From the initial pH of 8.0, it was observed that isolate BGS2 could reduce the pH up to 3.5 in modified thiosulphate broth which was in conformity with the findings of Kelly and Wood [18]. No reduction was found in NCL broth and Starkey broth (Table 2 and Plate 4). BGS2 isolate has been selected and purified for further studies.

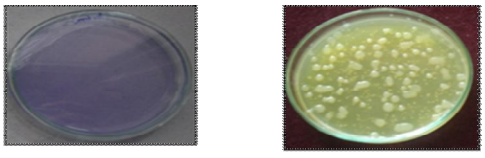
Table 2. Screening of sulphur oxidizing bacteria by pH reduction in the growth media (Initial pH: 8.0) (48 hrs incubation)

Name of isolate	Starkey broth	NCL broth	Modified Thiosulphate broth
Control	8.0	8.0	8.0
SWG1	8.0	8.0	4.7
SWG2	8.0	8.0	4.5
TRY1	8.0	8.0	4.3
TRY2	8.0	8.0	4.0
TRY3	8.0	8.0	4.4
BGS1	8.0	8.0	4.2
BGS2	**8.0**	**8.0**	**3.5**
BGS3	8.0	8.0	4.1
ALR1	8.0	8.0	5.0

Plate 4. pH reduction by Sulphur oxidizing bacterial isolates in broth assay



pH reduction by BGS2 isolate in modified thiosulphate agar medium



C –Control 1 - BGS2 2 - TRY2 3- SWG2
4- TRY1 5 – TRY3 6- BGS1 7 - BGS3
8- SWG1 9- ALR1

Production of sulphate by sulphur oxidizing bacterial isolates

The sulphate production by nine isolates of sulphur oxidizing bacteria was estimated. The isolate BGS2 recorded the highest sulphate production of 79.2 mg 100 ml^{-1} broth followed by TRY2 isolate which registered 68.80 mg 100 ml^{-1} broth. Teske *et al.* [19] reported that Sulphate production from thiosulphate over 20 days of aerobic incubation at 15^0C was found to be 2 to 4.6 mM sulphate which was in line with the present findings. The lowest sulphate production of 42.30 mg 100 ml^{-1} was recorded by ALR1 isolate. The results are presented in Fig. 1.

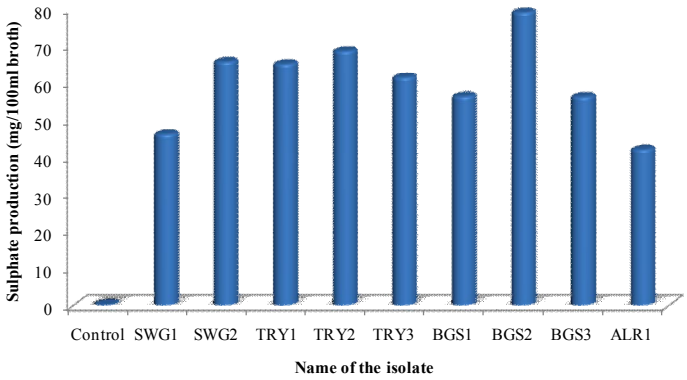


Fig. 1. Sulphate Production by SOB Isolates

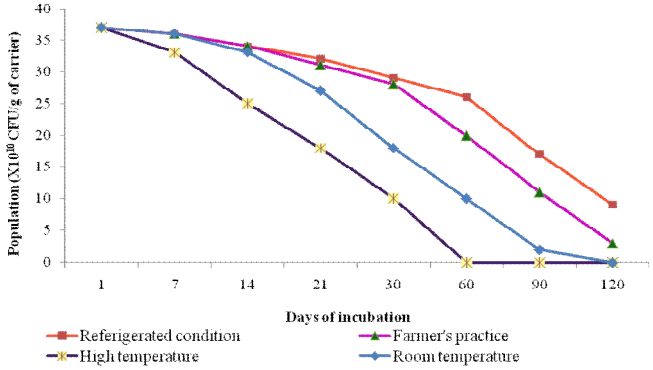


Fig2. Survival of BGS2 Isolate in Carrier Formulation During Different Incubation Temperature.

Studies on the development of formulation of sulphur oxidizing bacteria (BGS2 isolate)
Standardization of carrier based powder formulation of BGS 2

Carrier is a medium matrix on which the inoculated microorganisms grow to a reasonably higher population for an initial period and there after decline. So, there is a need to evaluate the survivability of inoculated microorganisms in the carrier. The present work reported that surviablity and enhancement of survivability of microorganisms with amendments rock phosphate and sodium thiosulphate.

The pH of the lignite was found to be 3.7. Addition of rock phosphate to lignite increased the pH. The ratio of lignite and rock phosphate at 1:1 ratio had pH of 6.4 which was near neutral pH (Table 3). Calcium Carbonate was added to make the pH of the carrier nearest to the neutral and similar combination was reported by Vimala and Sridar [20].

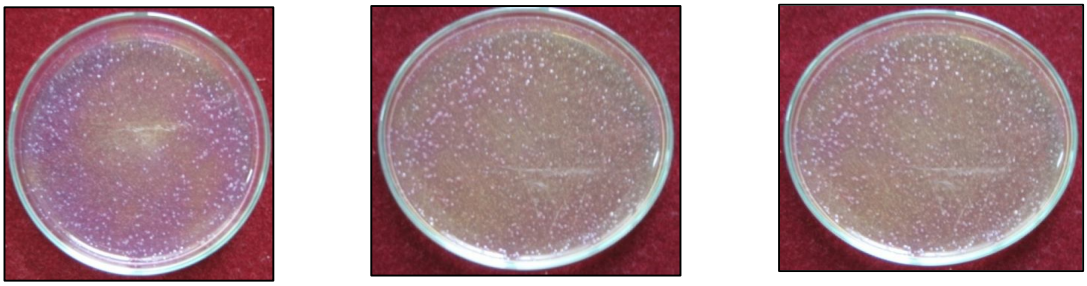
Table 3. Standardization of formulation for suitable pH

Lignite	Rock phosphate	pH
100%	-	3.7
90%	10%	4.4
80%	20%	4.7
70%	30%	5.4
60%	40%	6.0
50%	50%	6.4
-	100%	8.4

Amendment of sodium thiosulphate to the carrier improved the survival of BGS2 isolate in the carrier formulation. The results shown lignite carrier amended with 5% sodium thiosulphate supported the viability of BGS2 upto 90 days with 12 X 10^{10} cfu g^{-1} whereas the control recorded 5 X 10^{10} cfu g^{-1} up to 30 days (Table 4; Plate 5, 6). Similar enhancement of survivability of BGS2 by sodium thiosulphate amendment was reported by Vimala and Sridar [20]. Also, Fouilleux *et al.* [21] reported that significantly higher number of viable cells was enumerated from clay and rice bran + RP amended with 1% glucose than 0.1%. Reasonably, Gaind and Gaur [22] reported that a decline in population on prolonged incubation may be attributed to the depletion of nutrients, moisture and autolysis of cells.

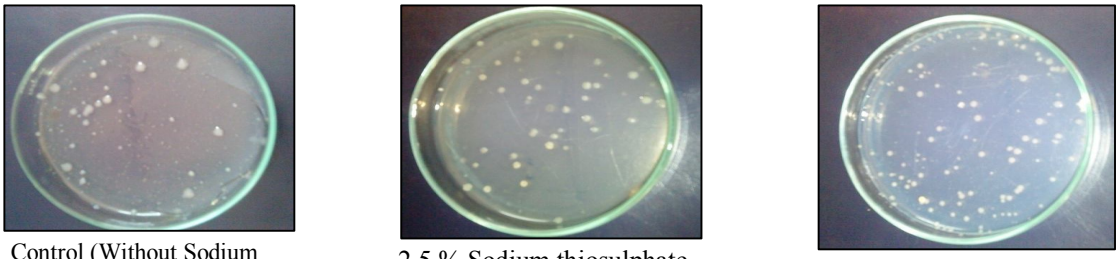
Table 4. Standardization of sodium thiosulphate on development formulation

Days	BGS2 population in carrier ($X10^{10}$ $cfug^{-1}$ of carrier)		
	Without Sodium Thiosulphate	2.5%	5%
0	49	49	49
7	40	45	47
14	32	37	44
21	19	24	40
30	5	11	34
60	-	2	21
90	-	-	12



Control (Without Sodium thiosulphate) | 2.5 % Sodium thiosulphate | 5 % Sodium thiosulphate

Plate 5. Influence of Sodium thiosulphate on the growth of BGS2 (Initial population)

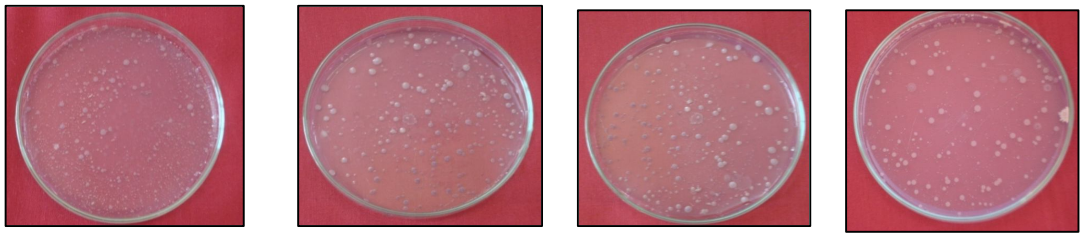


Control (Without Sodium thiosulphate) | 2.5 % Sodium thiosulphate | 5 % Sodium thiosulphate

Plate 6. Influence of Sodium thiosulphate on the growth of BGS2 (on 30th day)

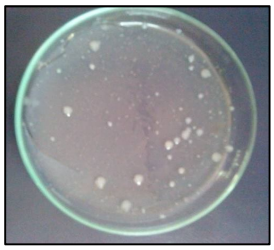
Influence of different incubation temperature on the shelf life of formulation

Temperature has greater influence on the survivability of microorganisms. The present study revealed that the population was found on 90 days after storage in room temperature (28°C) (2 X 1010 $cfug^{-1}$), refrigerated condition (4°C) (17 X 10^{10} cfu g^{-1}) and farmers practice (11 X 10^{10} $cfug^{-1}$) (Fig.2; Plate 7,8). The survival of population in high temperature (42° C) was only upto 30 days.The population was found to be decreasing during storage period and lower temperature has better survivability than higher temperature.

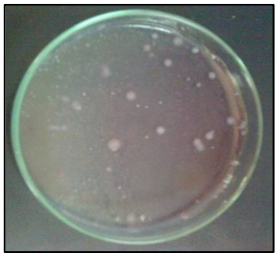


Refrigerated condition (4°C) | Farmer's practice | Room temperature (28°C) | High temperature (42°C)

Plate 7. Survival of BGS2 in the formulation at different temperature on 21st day.



Refrigerated condition (4°C)



Farmer's practice

Plate 8. Survival of BGS2 in the formulation at different temperature on 120th day

The report by several authors confirmed the findings of present investigation. Brockwell [23] observed that up to 40°C, there was no serious mortality, but beyond this temperature mortality rate was very high. Iswaran *et al.* [24] was reported that counts of *R. japonicum* at temperature ranging from 28 to 35°C were appreciable, while at a temperature of 40°C the mortality rate was high. The higher temperatures affect the longevity of transformant in all carriers [7].

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