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Co-Inoculation Effect of *Pseudomonas* and *Paenibacillus* On The Enhancement of Seed vigour Index In Tomato

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ABSTRACT
Tomato (Lycopersicon esculentum L) is one of the most popular as well as one of the most important vegetable crops in the tropic and temperate region. Rich in vitamin C and minerals especially phosphorus, potassium and calcium. Plant growth promoting rhizobacteria (PGPR) are bacteria that colonize tomato roots and encourage growth. The present research work has been undertaken with an aim to select the most efficient PGPR organisms viz., Pseudomonas fluorescens (PS-8) and Peanibacillus polymyxa (PA-8) from the rhizosphere of tomato and use the same maximisation of seed vigour index in tomato. Thesis the first comprehensive report on the positive role of co-inoculation of efficient Psudomonas fluorescens (PS-8) and Paenibacillus polymyxa (PA-8) viz., Growth phase, culture media (Nitrogen free medium), different inoculum level, growth temperature and the pH recorded highest seed vigour index in tomato when compared to other treatments.
Keywords: *Co-inoculation, Tomato, Psudomonas fluorescens, Paenibacillus polymyxa, Seed vigour index.*

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INTRODUCTION
Tomato (*Lycopersicon esculentum* L.) is one of the most important and widely grown vegetable crop in semi-arid condition. The cultivation of the same has become increasingly popular since mid-nineteenth century because of its varied climatic tolerance and high nutritive values. The fruits are rich source of vitamins, minerals and organic acids. Tomato is known for its outstanding nutritive value. It contains 93.1g of moisture, 1.9g of protein, carbohydrates 3.6mg, minerals 0.6g, fiber 0.7. Besides it contains Sodium, Potassium, Sulphur, Chlorine, Calcium, Magnesium, Phosphorus, Iron,Vitamin A,Thiamine, Riboflavin and Vitamin C, (31 mg per 100g of edible portion). Tomato can be used in fresh and processed forms, such as, salad, processed foods, like, ketchup, paste, soup, syrup, juice etc. Now-a-days, cultivation of tomato is the focus of horticultural industry in the world and takes a distinct place in the realm of vegetable crops [16]. In India, it is grown in an area of 0.5 M ha with an annual production of 7.4 MT and productivity of 14.3 tones ha^{-1}[17]. In Tamil Nadu, tomato is cultivated in area of 21,055 ha in the districts of Theni, Madurai, Coimbatore, Dharmapuri Erode and Salem district but the productivity is volatile [2].
An agricultural bioinoculant is a formulation containing one or more bacterial strains or species in an easy-to-use form. Higher degree of stress tolerance, long shelf life, enhanced

survivability in soils and on seeds and consistent plant response to inoculation are the important characteristics of any agricultural bioinoculant Neyra [21]. Okon [25] suggested the importance of the physiological status of microorganisms in agricultural bioinoculant preparation rather than the cell numbers to ensure more survival in carriers, survival in soil and on seed, colonization in the rhizosphere and positive plant response to bioinoculation. Neyra *et al.* [21] proposed the use of flocculated cell forms of *Azospirillum,* as delivery system, for the enhancement of growth and yield of crop plants under stresses. They described that *Azospirillum* biofloc contained high cell titre, increased adhesiveness to plant roots, enriched in encysted cells with thick capsules surrounded by EPS rich network which provided higher stress tolerance and longer shelf life to bioinoculant. Later, Neyra *et al* [22] proposed the concept of "Intergeneric microbial coaggregates" for the production of multipurpose agricultural bioinoculant with multiple benefits and developed *Azospirillum – Rhizobium* coaggregates for the enhancement of growth and yield in faba been (*Vicia faba*). Nikitina *et al.,* (2000[24]) confirmed the intergeneric coaggregation of *Azospirillum* with *Micrococcus, Bacillus* and *Rhizobium* sp., under *in vitro* condition.

vanVeen *et al.* [23] critically reviewed the reasons for the poor performance of agricultural bioinocula in natural environments and in rhizosphere of host plants. They suggested that instead of trying single strain with a single trait as agricultural bioinoculant trying to use microbial consortia for multiple benefits that also could thrive together in unique ecological nizhes as an ideal proportion. However, the performance of PGPR *viz.*, *Psudomonas* and *Paenibacillus* to various environmental stresses has not been exploited, so far.

Pseudomonas (PS-8) and *Paenibacillus* (PA-8) are the two important PGPR genera that are most frequently encountered from the rhizosphere of tomato crop plant [1, 19]. The PGPR characteristics of the genus *Pseudomonas* has already been well documented [20, 14, 18]. The gram-positive spore forming bacteria *viz., Paenibacillus sp.*, a phylogenetic variant of the genus *Bacillus* [3] has been described as an effective PGPR [8] and the ubiquitous occurrence of the same from the rhizosphere of tomato has already been reported [1].

However, there were no earlier reports on the comparative performance of different bioformulations of *Pseudomonas* and *Paenibacillus* cells, to various environmental stresses, available. Hence, the present study has been undertaken with an aim to exploit the comparative performance of co-inoculation *Pseudomonas* (PS-8) and *Paenibacillus*(PA-8) cells to Growth phase, culture media (Nitrogen free medium), different inoculum level, growth temperature and the pH recorded highest seed vigour index in tomato when compared to other treatments.

MATERIAL AND METHODS

Culture condition of *Pseudomonas* and Paenibacillus

The efficient strains of *Pseudomonas* (PA-8) and *Paenibacillus* (PA-8), isolated from the rhaizophere of tomato, grown at Keerapalaiyam, Cuddalore district, Tamil Nadu state, India, were used in the present study. The *Pseudomonas* and *Paenibacillus* cells were maintained in kings B broth and nutrient glucose agar slants, respectively, and incubated at 28 ± 2◦C, with monthly transfer.

Preparation of inoculum

The PGPR strains of *Pseudomonas* and *Paenibacillus* isolates were individually grown in base-77 and nutrient glucose broth, respectively, in a shaking bath at 28 ± 2◦C for 24 hr. Then, the media were centrifuged separately at 5000× g for 10 min to harvest the log phase cells of the above stains. The pellets were washed three times with 0.1M phosphate buffer (pH 6.8), individually. Finally, the cells of *Pseudomonas* and *Paenibacillus* were resuspended, separately, in the same buffer at a cell concentration of 1×10^7 CFU/mL by measuring the OD at 420 nm for *Pseudomonas* and 540 nm for *Paenibacillus* and used as inoculum source.

Preparation of Co-Ag buffer [10]

The co-inoculation buffer was prepared as stated below according to Grimaudo and Nesbitt (1997[10]).

Estimation of co-inoculation Percentage

The co-inoculation percentage of *Pseudomonas* and *Paenibacillus* cells were done according to the procedure of Madi and Henis [13]

Preparation of co-inoculation of *Pseudomonas* and *Paenibacillus* cells

One mL aliquot of each PGPR strains (1 x 10^7 cells mL-1) was mixed together in 10 mL of Co-Ag buffer Grimaudo (1997). The mixture was vortexed for 10 sec, shaken on a rotary platform shaker for 3 min and left undisturbed at room temperature for, 1h.

Factors affecting co-inoculation of *Pseudomonas* and *Paenibacillus* on the enhancement seed viguor index of tomato (*var.* PKM – 1)

Tomato (L*ycopericon esculentum* L.) *c.v.* PKM-1 tomato seeds were surface sterilized by immersion in 95 per cent ethanol for 1 min, followed by 20 min in 1 per cent Na OCl. After rising three times with sterile distilled water, the sterilized seeds were placed on the surface of 1 per cent water agar in Petri plates (9 cm dia, at five seeds per plate). Then, they were incubated in an inverted position for 3 days at room temperature to allow germination. The plates were sealed with wax to avoid agar dryness during germination.

The *Pseudomonas* and *Paenibacillus viz.,* PS-8 and PA-8 were grown individually in King' s B broth and nutrient glucose broth, respectively, maintained in a shaking bath at 30 ± 2^0 C.

The tomato seeds were subjected to treatments, dried in shade for 30 min. then, the inoculated tomato seeds were arranged in two rows on a sheet of blotting paper dipped in sterile water. Then, they were covered with another blotting paper dipped in sterile water, rolled and placed vertically in a moist chamber at 20^0 C. uninoculated seeds with distilled water treatment served as control. After the incubation for 5 days, each roll was opened and the vigour indices of germinated tomato seeds were calculated by the method:

Vigour index = (mean root length + mean hypocoty length) × % germination

Co-inoculation effect of *Pseudomonas* and *Paenibacillus* cells on the enhancement of seed vigour index of tomato. (PKM – 1)

Growth phase, culture media (Nitrogen free medium), different inoculum level, growth temperature and the pH of *Pseudomonas* (PS-8) and *Paenibacillus* (PA-8) co-inoculation were done according to Madi and Henis [13].

Statistical analysis

The experimental results were statistically analyzed in randomized block design (RBD) and in Duncan's multiple range test (DMRT) as per the procedure.

RESULTS AND DISCUSSION

In the present study, the efficient PGPR cells *viz., Pseudomonas* (PS-8) and *Paenibacillus* (PA-8), *viz.,* inoculum level, growth phase, culture media, temperature and pH level, on the enhancement of seed vigour index of tomato, during co- inoculation was studied and the results are discussed hereunder.

The effect of different growth phases *viz.,* lag, log and stationary on the enhancement of seed vigour index of tomato during the co-inoculation of PGPR cells *viz.,* PA-8 and PS-8 was studied. Growth phase of the microorganisms played a critical role on the co-inoculation processes. Among the different growth phases tested, co-inoculation of log growth phase of *Pseudomonas* and *Paenibacillus* cells recorded the highest seed vigour index followed by lag and stationary growth phases of *Pseudomonas* and *Paenibacillus* cells. The lowest seed vigour index was recorded with the stationary growth phase of PGPR cells and revealed the fact that active metabolic state of the microbial cell was conducive for co-inoculation processes. The effect of the culture age on the composition of the cell surface of bacteria has been reported by Burdman *et al.* [4], Nikitiana *et al.* [24] The results of the present study clearly revealed the determined role of growth phase of *Pseudomonas* and *Paenibacillus* cells on the seed vigour index of tomato and also in conformity with the earlier findings.

The effect of different cultural conditions *viz.,* N-free and N-supplemented of efficient *Pseudomonas* and *Paenibacillus* cells *viz.,* PS-8 and PA-8, on the enhancement of seed vigour index of tomato was studied under *in vitro* condition. Cultural conditions of the microorganisms played a key role in determining the coinoculation processes. Between the two cultural conditions studied, namely, *Pseudomonas* and *Paenibacillus* cells grown in N-free medium and *Pseudomonas* and *Paenibacillus* cells grown in N-supplemented medium, the PGPR cells, collected from N-free medium, recorded more seed vigour index than the *Pseudomonas* and *Paenibacillus* cells, collected from N-supplemented medium. Kolenbrander [12] summarized the effect of culture medium on the flocculation of *Streptococcus* and *Actinomyces* suspensions, collected from human oral ecosystem. The

results of the present study revealed the highest seed vigour index of tomato by *Pseudomonas* and *Paenibacillus* cells, collected from N-deficient medium, which affected the cell surface characteristics of *Pseudomonas* and *Paenibacillus* cells and finally resulted in higher seed vigour index of tomato.

The effect of different level of growth temperature *viz.,* 25, 30, 35, 40 and 45°C of *Pseudomonas* and *Paenibacillus* cells *viz.,* PS-8 and PA-8 maintained in growth medium was studied on the enhancement of seed vigour index during coinoculation. The growth temperature level of *Pseudomonas* and *Paenibacillus* cells played a critical role in determining the seed vigour index of tomato. The increasing level of growth temperature of *Pseudomonas* and *Paenibacillus* cells upto 35°C increase the seed vigour index of tomato and thereby a reduction in the same was recorded. Ketstrup and Funder-Nielsen [9] reported the positive effect of growth of temperature in determining the virulence of *Streptococcus* with *Fusobacterium* and *Actinomyces*. Burdman *et al*. (1998) reported the positive effect of growth temperature on cell surface protein of *Azospirillum brasilense* cd cells. The results of the present study are also in conformity with the above findings.

The effect of different levels of buffer pH of *Pseudomonas* and *Paenibacillus* cells *viz.,* PS-8 and PA-8 was studied on the enhancement of seed vigour index of tomato during co-inoculation. The pH level of growth medium exerted a positive role on the virulence in order to attain maximum seed vigour index of tomato. Among the different buffer pH levels tested, the 7.0 level of buffer pH, recorded the highest seed vigour index followed by 7.5, 6.5, 6.0 buffer pH levels. Madi and Henis [13] reported the positive effect of pH on virulence of *Azospirillum* cells and Burdman *et al*. [4] reported the involvement of charged groups in this phenomenon and the *Azospirillum* strains Cd and FAJ 0204 responded differentially to the levels of pH. They also added that the negative ionized groups of bacterial cell surface could be neutralized by protonation thus reducing the strength of repulsive forces between bacteria and leading to coinoculation Processes The results of the present study also revealed the differential response of *Pseudomonas* and *Paenibacillus* cells to different pH levels and in conformity with the above findings.

Table – 1: Coinoculation Effect of *Pseudomonas* and *Paenibacillus* cells, at Different Growth Stages, on the Enhancement of Seed Vigour Index of Tomato. (PKM – 1)

Isolate[a]	Growth phase	Seed vigour index[b]	Statistics[c]
PS-8: PA-8	Lag	14148 ± 13.64	b
	Log	15874 ±17.29	a
	Stationary	13456 ± 11.84	c
LSD (P<0.05)		**0.98**	

a - Growth phase of PGPR cells *viz.*, *Pseudomonas* and *Paenibacillus* , individually cultured and collected from King' s B broth and glucose broth, respectively, at different growth phase and used for co inoculation experiment at 10^7 : 10^7 inoculum level

b -Values are mean of three replication ± SD

c -Values followed by different letters are significantly differed at 5% level according to student 't' test

Table-2: Coinoculation Effect of *Pseudomonas And Paenibacillus* Cells, Cultured in Different Growth Media, on the Enhancement of Seed Vigour Index of Tomato (PKM – 1)

Isolate	Culture Medium[a]	Inoculum Level CFU / mL^{-1}	Seed vigour index[c]	Statistics[d]
PS-8 :PA-8	DFMM: GB[a]		14678 ± 16.78	a
	King's B: NGB	10^7 : 10^7	13654 ± 12.78	b
LSD (P<0.05)			**0.96**	

a - DFMM – Dworkin and Foster minimal salts medium; GB – Glucose broth; NGB – Nutrient glucose broth; King' s B broth

b - Medium from which PGPR cells are harvested at stationary phase and utilized for co inoculation experiment

c - Values are mean of three replication ± SD

d - Values followed by different letters are signicantly differed at 5%

Table - 3: Coinoculation Effect of *Pseudomonas* And *Paenibacillus* Cells, at Different Inoculum Level, On The Enhancement of Seed Vigour Index of Tomato (PKM – 1)

Isolate[a]	Inoculum level[b] (CFU / mL$^{-1)}$	Seed vigour index	Statistics[c]
PS-8:PA-8	10^4 :10^4	10056 ± 5.54	e
	10^5 : 10^5	12453 ± 9.78	d
	10^6 : 10^6	13765 ± 10.65	c
	10^7 : 10^7	14876 ± 13.67	a
	10^8 : 10^8	14134 ± 12.54	b
LSD(P<0.05)	**0.93**		

a- Growth phase of PGPR cells *viz.*, *Pseudomonas* and *Paenibacillus* cells, individually cultured rom King's B broth and glucose broth, respectively, used for coinoculation experiment

b - Values are mean of three replication ± SD

c -Values followed by different letters are significantly differed at 5% level according to student's' test

Table-4: Coinoculation Effect of *Pseudomonas* and *Paenibacillus* Cells, Grown At Different Levels Of Ph, On The Enhancement Of Seed Vigour Index Of Tomato (PKM – 1)

Isolate, culture medium and Growth phase[a]	pH level of buffer	Seed vigour index[b]	Statistics[c]
PS-8 – DFMM – Stationary PA-8 – GB –Stationary	6.0	12654 ± 9.87	d
	6.5	13565 ± 10.87	c
	7.0	14876 ± 13.65	a
	7.5	14127 ± 12.43	b
LSD (P<0.05)		**0.97**	

DFMM - Dworkin foster minimal salts medium; GB – Glucose broth

a - PGPR cells *viz.*, *Pseudomonas* and *Paenibacillus*, at inoculums level of 10^7:10^7 CFU mL^{-1}

b - Values are mean of three replication ± SD

c -Values followed by different letters are significantly differed at 5% level according to student's' test

Coinoculation of *Pseudomonas* and *Paenibacillus* cells 25,30,35,40,45 temperature level augment more seed vigour index tomato than other temperature levels.

25,30,35,40,45 temperature level recorded the highest seed vigour index in tomato (14765) when compared results to other temperature levels. The results are statistically significant at 5% level and the results presented in (Table-5).

Table-5: Co inoculation Effect of *Pseudomonas* and Paenibacillus Cells, Grown at Different Levels of Temperature, on The Enhancement of Seed Vigour Index of Tomato (PKM – 1)

Isolate, culture medium and Growth phase[a]	Growth Temperature (^{0}C)	Seed vigour index[b]	Statistics[c]
PS-8 – DFMM – Stationary PA-8 – GB – Stationary	25	10143 ± 6.89	e
	30	12345 ± 9.68	d
	35	14765 ± 17.54	a
	40	14568 ± 12.67	b
	45	13453 ± 15.54	c
LSD(P<0.05)		**0.96**	

DFMM - Dworkin foster minimal salts medium; GB – Glucose broth

a - PGPR cells *viz., Pseudomonas* and *Paenibacillus* at inoculums level of 10^7:10^7 CFU mL^{-1} and at 7.5 pH of buffer level

b - Values are mean of three replication ± SD

c - Values followed by different letters are signicantly differed 5% level according to student 't' test

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