ISSN 1392-3196 / e-ISSN 2335-8947
Zemdirbyste-Agriculture, vol. 109, No. 2 (2022), p. 139–148
DOI 10.13080/z-a.2022.109.018

# Statistical production optimisation, *Fusarium* biocontrol and insecticidal activity of 2,4-diacetylphloroglucinol produced by a newly discovered moderately haloalkalitolerant *Pseudomonas guariconensis* VDA8

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## Abstract

A newly discovered rhizobacterial strain, *Pseudomonas guariconensis* VDA8, was observed to secrete antimicrobial 2,4-diacetylphloroglucinol (DAPG). The strain is characterised by plant-growth promoting (PGP) traits of indole acetic acid, siderophore, exopolysaccharide, ammonia and lytic enzyme production, phosphate solubilisation, in addition to being salt (0.85 M) and alkali (pH 8.5) tolerant. Optimisation for maximal DAPG production was investigated using statistical tools viz. Plackett-Burman design (PBD) and central composite design (CCD). Out of 19 nutritional ingredients of Schlegel's synthetic medium and physico-chemical factors, aeration and pH were discovered to be most influential by a 20-run PBD trial. A full factorial CCD with five levels was conducted using 13 experimental runs in modified Schlegel's synthetic medium to reveal the aeration rate of 144 rpm and pH 8.9 as optimal conditions for DAPG production of 1.4 g $L^{-1}$ at shake flask level. The 1 L volume fermentation for validation of the CCD model resulted in yield of 1.26 g $L^{-1}$ to reveal that media volume and initial cell density are also crucial factors for DAPG production by *P. guariconensis*. Further, seed biopriming of chickpea (*Cicer arietinum* L.) demonstrated improved germination (93.3%) and vigour index (4.0) compared with non-primed seeds. The biocontrol activity examined in pot bioassay against phytopathogen *Fusarium oxysporum* recorded highest germination (95.5%), seedling vigour, and plant growth indices as compared to DAPG (30 µg $ml^{-1}$) chemoprimed, proving *P. guariconensis* VDA8 has a combined PGP and biocontrol activity. Moreover, purified DAPG (1 mg $ml^{-1}$) exhibited pest repellence and insecticidal activity against food storage pests *Sitophilus oryzae* and *Rhyzopertha dominica* adults with a mortality of 55–60% in chemoprimed rice after 72 h. Direct contact toxicity was recorded to reach 66–70% within 48 h at similar concentration.
The present study strongly advocates the use of *P. guariconensis* VDA8 as a dual PGP and biocontrol agent in saline and alkaline soil as well as purified DAPG as potent insecticide for post-harvest grain preservation.

Keywords: Plackett-Burman design, central composite design, *Cicer arietinum*, *Fusarium oxysporum*, *Sitophilus oryzae*, *Rhyzopertha dominica*.

## Introduction

Climate change, the growth of human population, and food demand are posing a challenge to increase crop productivity. In order to deal with seasonal availability of rainwater, often heavy use of groundwater rich in salts and heavy metals is extensively used for crop irrigation. This has led to depleting groundwater, a decrease in available fertile land, and damage to the soil ecosystem, mainly the microbiota. To increase yield and protect crop produce from insect pests during storage, farmers often use chemical fertilisers and pesticides. These measures are temporarily effective, expensive, difficult to use, dangerous to farmers, and deleterious to soil ecosystem. Moreover, chemical leaching to the food, feed, groundwater, and streams, resistance in phytopathogens and insect pests has posed global health and environment concerns. According to the FAO world map (FAO, 2021a), globally about 8.7%, or 1.257 billion ha, is salt-affected soil. Additionally, the extremes of acid or alkaline pH severely affect micro- and macroflora, thereby decreasing soil fertility and posing food insecurity. Soil management practices to remediate salinisation and sodicity necessitate selection, development, and the use of indigenously adapted salt- and extreme pH-tolerant plant-associated microbial inputs. On the other hand, the last three decades have seen prodigious use of chemical-based agricultural inputs raising severe environmental and health concerns.

An astounding 20–30% of annual agri-produce is lost due to ~70,000 pest species and ~10,000 fungal-phytopathogens at pre-/post-harvest phases (FAO, 2017 a; b; Davies et al., 2021). Generally, fungal diseases may be overcome by the reduction of the inoculums, inhibition of its virulence mechanisms, and promotion of genetic diversity in the crop (Strange, Scott, 2005). However, instead of natural organic inputs, a stupendous quantity of chemical pesticides is used. Over a decade, average global pesticide use was ~41 million tonnes per annum (910,000 t of fungicides and bactericides and 680,000 t of insecticides) and expected to increase by 4.7% during the next five years (FAO, 2021b). Although the use of chemicals significantly reduces fungal infection of crop and produce, it has caused concerns about food chain penetration, acute and chronic toxicity threat to living beings, and emergence of fungicide resistance in phytopathogens (Davies et al., 2021). Alternatively, the use of natural bioactive antagonistic compounds to control soil-borne

Please use the following format when citing the article:
Kankariya R. A., Chaudhari A. B., Dandi N. D. 2022. Statistical production optimisation, *Fusarium* biocontrol and insecticidal activity of 2,4-diacetylphloroglucinol produced by a newly discovered moderately haloalkalitolerant *Pseudomonas guariconensis* VDA8. Zemdirbyste-Agriculture, 109 (2): 139–148. DOI 10.13080/z-a.2022.109.018

pathogens is emphasised and has gained increased attention for sustainable agriculture.

2,4-diacetylphloroglucinol (DAPG) is a phenolic polyketide secreted by most of the plant-associated *Pseudomonas* spp. that control phytopathogens and suppress plant diseases (Kankariya et al., 2019). However, genes for DAPG production are confined to few strains such as *P. fluorescens*, *P. protegens*, *P. chlororaphis*, and *P. brassicacearum* isolated globally from disease suppressing soils (Gross, Loper, 2009; Zhou et al., 2012; Gutiérrez-García et al., 2017). The use of these strains for fermentative bacterial DAPG production is attractive, but yields are often meagre and, therefore, do not attract industrial-scale production. On the other hand, direct field application of live Pseudomonads is uneconomical due to: (1) uncontrolled prorogation environment, (2) liquid phase, (3) non-sporulating physiology, and (4) short shelf-life. To circumvent this, farmers can be encouraged for field-level production, but the need for training to acquire specialised microbiological skills, the use and availability of equipment, tedious and inconsistent production performance of Pseudomonads, and quality assessment are impractical. Also, it could be expensive for personnel and hazardous for environment. Eventually, DAPG-producing *Pseudomonas* biocontrol agents have largely failed to raise interest in industries to leap from lab-to-land despite potential plant-growth promoting (PGP) benefits.

Notably, optimal fermentative DAPG production depends on a variety of biotic and abiotic factors, e.g., genetic content, environmental availability of nutrients, metabolic rate and growth phase, interaction with other organisms, host factors such as root exudates, and cultivar. The PGP and biocontrol performance is also greatly affected by the physico-chemical quality of the soil and is particularly challenging if the soil is saline and/or alkaline. Therefore, biofertilising and biocontrol microbial agents experience lack of adaptability and natural tolerance to a variety of stress conditions.

It is also imperative to understand the key nutritional principles of DAPG-producing *Pseudomonas* in the laboratory before its use in the field for optimal performance. Thus, the first step to the production of fermentative DAPG mandates a selection of multi-stress tolerant *Pseudomonas* strain(s) followed by the optimisation of fermentative media with respect to composition and bioprocess parameters, viz. (1) carbon sources and their ratios, (2) nitrogen (organic and inorganic) sources, (3) phosphate levels, (4) trace minerals, and (5) environmental conditions viz. pH, temperature, and oxygen transfer (Duffy, Defago, 1999; Saharan et al., 2011). For this purpose, production optimisation using the laborious one-variable-at-a-time (OVAT) approach is less preferred over the use of more accurate statistical tools to screen significant process variables, which is often followed by full-factorial optimisation of the significant factors and a trial run with optimised parameters for model validation. Yet, as per our literature survey, there are very few scientific studies that have undertaken such efforts to produce DAPG at industrial scale. Previously, we explored for a multi-stress tolerant (salt and alkaline stress) DAPG producing PGP microbe(s), isolated, and screened moderate haloalkalitolerant (5% NaCl, pH 8.0) *Pseudomonas guariconensis* strains from cotton rhizospheric soil detected to produce DAPG (unpublished data).

With this background, the aim of the study was to (1) optimise for maximal DAPG production by *P. guariconensis* VDA8 with respect to nutritional ingredients of Schlegel's synthetic medium and physico-chemical factors using statistical tools, (2) assess DAPG biopriming and biocontrol activity against phytopathogenic *Fusarium oxysporum* in chickpea, and (3) examine the insecticidal effects of purified DAPG on treated grain. Overall, as per our literature survey, the present study demonstrates first efforts with respect to DAPG for (1) amenability for use of a novel *P. guariconensis* for DAPG fermentation at pilot-scale, (2) antagonistic activity against phytopathogenic *F. oxysporum* in chickpea, and (3) a potential avenue to prevent post-harvest loss of grain from the common insect pests viz. *Sitophilus oryzae* and *Rhyzopertha dominica*.

# Materials and methods

All the chemicals, solvents, and culture media used in this study were of the highest purity, procured from Hi-Media Laboratories (India), Merck (Germany), and Qualigens Fine Chemicals (India), and of analytical reagent (AR) grade. Similarly, all glassware was cleaned with 6 N HCl to eradicate residual metals and ions, washed several times with distilled water, and dried in an oven before use.

*Microorganisms, insects and culture conditions.* Isolated from the cotton rhizospheric soil, the Gram-negative plant-growth promoting (PGP) rhizospheric bacterial strain *Pseudomonas guariconensis* VDA8 (GenBank accession No. MN577431) was maintained on King's B (KB) or nutrient agar slants at 4°C. The strain was putatively characterised to produce 2,4-diacetylphloroglucinol (DAPG) in Schlegel's synthetic medium under non-optimised conditions. The fungal phytopathogen *Fusarium oxysporum* MTCC 9913 was obtained from the Microbial Type Culture Collection (MTCC) and Gene Bank and maintained on potato dextrose agar at 28°C by regular sub-culturing at bimonthly intervals. The insects *Sitophilus oryzae* and *Rhyzfopertha dominica* were nurtured on rice grains supplemented with 5% yeast extract in the laboratory enclosure at 25 ± 1°C with 65 ± 5% relative humidity. For the bioassay, two-week-old adults of *S. oryzae* and *R. dominica* were used. Reference DAPG was procured from Toronto Research Chemicals Inc., Canada.

*Optimisation of nutrient medium components by Plackett-Burman design (PBD) and of significant variables using central composite design (CCD).* From one-variable-at-a-time (OVAT) analysis, 19 variables ($k = 19$) including (1) carbon sources, i.e., glycerol and molasses (equivalent to reducing sugar content), (2) nitrogen sources i.e., ammonium chloride and urea, (3) microelements, i.e., disodium orthophosphate ($Na_2HPO_4$), sodium dihydrogen phosphate ($NaH_2PO_4$), potassium chloride (KCl), magnesium sulphate ($MgSO_4$), calcium chloride ($CaCl_2 \times 2H_2O$), zinc sulphate ($ZnSO_4 \times 7H_2O$), cobalt chloride ($CoCl_2 \times 6H_2O$), manganese chloride ($MnCl_2 \times 4H_2O$), copper chloride $CuCl_2 \times 2H_2O$), boric acid ($H_3BO_3$), nickel chloride ($NiCl_2 \times 6H_2O$), and sodium molybdate ($Na_2MoO_4 \times 2H_2O$), and (4) physico-chemical parameters, i.e., aeration (rotations per minute, rpm), incubation temperature (°C), and initial pH were selected for DAPG production by *P. guariconensis* VDA8. The medium components were screened using two-factorial experimental PBD by $n$ variables with $n + 1$ experiments (Table 1).

From PBD, the most significant variables ($n = 2$) with high $F$-value and $p \leq 0.05$ were further screened by the CCD employing response surface methodology (RSM). A full factorial CCD for two factors, i.e., aeration (rpm) and pH, each with five levels (–1.4142, –1, 0, 1, and 1.4142), was evaluated using 13 experimental runs to predict the optimum level for maximal DAPG production. There were four cube points, five centre points in cube, and four axial points; there were no centre points in axial.

Each experimental response was recorded as mean of three independent experiments, and the results were obtained as mean ±SD. Both PBD and CCD were evaluated using analysis of variance (ANOVA) by statistical software Minitab, version 16 (Minitab LLC, USA). The goodness of fit of the polynomial model equation was determined from the coefficient ($R^2$).

*Purification and characterisation of DAPG.* To validate the accuracy of the model, DAPG fermentation was carried out in a 5 L capacity flask with 1 L ml modified Schlegel's synthetic medium for a duration of 72 h at 30°C and 144 rpm aeration. For the separation of crude DAPG, the cell suspension was centrifuged at 8000 rpm for 15 min to collect cell-free supernatant. The pH of the supernatant was adjusted to 2.0 by the addition of 4N HCl before DAPG extraction (3×) with an equal volume of ethyl acetate in a separating funnel. The organic phase was evaporated to dryness at 45–55°C by using a rotavapor vacuum evaporator Büchi R-124 with waterbath B-480 (Switzerland). The extracted crude DAPG was dissolved in methanol and stored at −20°C before further purification and analysis. Purification was achieved by column chromatography using a glass column (50 × 2 cm) packed with a slurry of silica gel, 60–120 mesh size (HiMedia Laboratories, India) preactivated at 120°C (4 h) for adsorption (Reddy et al., 2007). The crude extract was re-dissolved in 20 ml of ethyl acetate and applied to the column. Later, the column was washed with 50 ml of ethyl acetate and eluted with 300 ml of toluene-acetone (4:1, v/v). Five-millilitre fractions were collected and examined for the presence of DAPG by thin-layer chromatography (TLC) in a UV chamber against the standard reference. The fractions of similar compositions were mixed for further studies.

***Table 1.*** Experimental design matrix of Plackett-Burman design (PBD) and results of the evaluation of variables affecting DAPG production by *Pseudomonas guariconensis* VDA8

| Run | Factor | | | | | | | | | | | | | | | | | | | DAPG production µg ml$^{-1}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S | |
| 1. | 15 | 5 | 3.8 | 0.9 | 0.28 | 0.3 | 0.4 | 0.4 | 0.7 | 0.01 | 0.02 | 0.03 | 0.001 | 0 | 0 | 0.003 | 120 | 25 | 10 | 76.77 ± 0.11 |
| 2. | 15 | 5 | 4.6 | 1.5 | 0.36 | 0.3 | 0.3 | 0.6 | 0.7 | 0.01 | 0.02 | 0 | 0 | 0 | 0 | 0.003 | 0 | 37 | 8 | 240.87 ± 0.40 |
| 3. | 9 | 5 | 4.6 | 0.9 | 0.36 | 0.3 | 0.4 | 0.6 | 1.1 | 0 | 0 | 0.03 | 0.001 | 0 | 0.002 | 0.003 | 0 | 25 | 8 | 795.07 ± 0.80 |
| 4. | 9 | 1 | 4.6 | 1.5 | 0.28 | 0.4 | 0.4 | 0.4 | 0.7 | 0 | 0.02 | 0 | 0.001 | 0 | 0.002 | 0.003 | 120 | 37 | 8 | 77.80 ± 1.06 |
| 5. | 15 | 1 | 3.8 | 1.5 | 0.36 | 0.4 | 0.4 | 0.4 | 1.1 | 0.01 | 0 | 0.03 | 0.001 | 0 | 0 | 0 | 0 | 37 | 8 | 258.47 ± 0.79 |
| 6. | 9 | 1 | 3.8 | 0.9 | 0.28 | 0.4 | 0.3 | 0.6 | 1.1 | 0.01 | 0.02 | 0.03 | 0 | 0 | 0.002 | 0.003 | 0 | 37 | 10 | 77.57 ± 0.11 |
| 7. | 15 | 1 | 4.6 | 1.5 | 0.28 | 0.3 | 0.4 | 0.6 | 1.1 | 0.01 | 0 | 0 | 0 | 0 | 0.002 | 0 | 120 | 25 | 10 | 490.27 ± 0.09 |
| 8. | 9 | 5 | 4.6 | 1.5 | 0.36 | 0.4 | 0.3 | 0.4 | 1.1 | 0 | 0.02 | 0.03 | 0 | 0 | 0 | 0 | 120 | 25 | 10 | 253.27 ± 1.32 |
| 9. | 9 | 1 | 4.6 | 0.9 | 0.36 | 0.4 | 0.4 | 0.6 | 0.7 | 0.01 | 0.02 | 0 | 0.001 | 0.03 | 0 | 0 | 0 | 25 | 10 | 102.55 ± 1.58 |
| 10. | 15 | 1 | 4.6 | 0.9 | 0.28 | 0.3 | 0.3 | 0.6 | 1.1 | 0 | 0.02 | 0.03 | 0.001 | 0.03 | 0 | 0 | 120 | 37 | 8 | 84.18 ± 0.25 |
| 11. | 15 | 5 | 3.8 | 0.9 | 0.36 | 0.4 | 0.3 | 0.6 | 0.7 | 0 | 0 | 0 | 0.001 | 0 | 0.002 | 0 | 120 | 37 | 10 | 118.88 ± 1.02 |
| 12. | 15 | 1 | 4.6 | 0.9 | 0.36 | 0.4 | 0.3 | 0.4 | 0.7 | 0.01 | 0 | 0.03 | 0 | 0.03 | 0.002 | 0.003 | 120 | 25 | 8 | 378.58 ± 0.68 |
| 13. | 15 | 5 | 3.8 | 1.5 | 0.28 | 0.4 | 0.4 | 0.6 | 0.7 | 0 | 0.02 | 0.03 | 0 | 0.03 | 0.002 | 0 | 0 | 25 | 8 | 639.17 ± 0.68 |
| 14. | 15 | 5 | 4.6 | 0.9 | 0.28 | 0.4 | 0.4 | 0.4 | 1.1 | 0 | 0 | 0 | 0 | 0.03 | 0 | 0.003 | 0 | 37 | 10 | 711.30 ± 0.74 |
| 15. | 9 | 1 | 3.8 | 0.9 | 0.28 | 0.3 | 0.3 | 0.4 | 0.7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 25 | 8 | 623.97 ± 0.62 |
| 16. | 15 | 1 | 3.8 | 1.5 | 0.36 | 0.3 | 0.3 | 0.4 | 1.1 | 0 | 0.02 | 0 | 0.001 | 0.03 | 0.002 | 0.003 | 0 | 25 | 10 | 156.29 ± 0.75 |
| 17. | 9 | 5 | 4.6 | 1.5 | 0.28 | 0.3 | 0.3 | 0.4 | 0.7 | 0.01 | 0 | 0.03 | 0.001 | 0.03 | 0.002 | 0 | 0 | 37 | 10 | 285.24 ± 1.29 |
| 18. | 9 | 5 | 3.8 | 1.5 | 0.28 | 0.4 | 0.3 | 0.6 | 1.1 | 0.01 | 0 | 0 | 0.001 | 0.03 | 0 | 0.003 | 120 | 25 | 8 | 173.22 ± 0.71 |
| 19. | 9 | 1 | 3.8 | 1.5 | 0.36 | 0.3 | 0.4 | 0.6 | 0.7 | 0 | 0 | 0.03 | 0 | 0.03 | 0 | 0.003 | 120 | 37 | 10 | 77.04 ± 0.37 |
| 20. | 9 | 5 | 3.8 | 0.9 | 0.36 | 0.3 | 0.4 | 0.4 | 1.1 | 0.01 | 0.02 | 0 | 0 | 0.03 | 0.002 | 0 | 120 | 37 | 8 | 332.24 ± 0.69 |

*Note.* Factors are coded as (g L$^{-1}$): A – glycerol, B – molasses (reducing sugar), C – $Na_2HPO_4$, D – $NaH_2PO_4$, E – $NH_4Cl$, F – urea, G – KCl, H – $MgSO_4 \times 7H_2O$, I – $CaCl_2 \times 2H_2O$, J – $ZnSO_4 \times 7H_2O$, K – $CoCl_2 \times 6H_2O$, L – $MnCl_2 \times 4H_2O$, M – $CuCl_2 \times 2H_2O$, N – $H_3BO_3$, O – $NiCl_2 \times 6H_2O$, P – $Na_2MoO_4 \times 2H_2O$, Q – aeration (rotations per min, rpm), R – temperature (°C), S – pH.

*Characterisation of DAPG.* The UV-Vis absorption spectrum of purified DAPG was recorded in the range from 200 to 800 nm wavelength, where methanol was used as a blank using Nanodrop UV-Visible spectrophotometer ND-1000 (Nanodrop Tech Inc., USA). Fourier transform infrared spectroscopy (FTIR) was used to determine the functional groups and the type of bond present in DAPG compound with spectrometer Spectrum Two (Perkin Elmer, USA). Purified DAPG was ground with spectral grade KBr (Merck, Germany) to fine powder at room temperature and pressed with 6000 kg cm$^{-2}$ for 120 s to obtain translucent KBr pellets, and the absorbance was recorded. TLC analysis of the purified DAPG was carried out using the solvent system (benzene:acetic acid; 95:05) on a TLC plate (20 × 20 cm) Silica gel 60 $F_{254}$ (Merck, Germany). The purified DAPG was subjected to high-performance liquid chromatography (HPLC) to determine the retention time of the purified sample with UV absorbance at 270 nm. The 20 µl samples were injected into the HPLC Isocratic System UV Detector (Younglin, South Korea), software Autochro-3000 equipped with a C18 reverse phase column (4.6 × 150 mm with 5 µm particle size packing V) at ambient temperature. The isocratic mobile phase was comprised of acetonitrile (0.1%): orthophosphoric acid (55:45 v/v) at a flow rate of 0.7 ml min$^{-1}$ for 10 min (Zhang et al., 2014). The purified DAPG was subjected to liquid chromatography-mass spectrometry (LC-MS) analysis using Waters 2795 HPLC coupled with hybrid Waters Quadruple orthogonal acceleration time of flight (Q-TOF) Micromass electrospray ionisation-mass spectrometry (ESI-MS) for molecular weight determination.

*Seed germination assay for chickpea.* To test the outcome of *P. guariconensis* VDA8 seed priming on germination, bacterisation was achieved as per Ownley et al. (2003). The bacterial cells were grown in KB medium, and cell pellets were harvested aseptically by centrifugation at 3000× *g*. Cell pellets were washed several times with sterile distilled water and suspended in sterile saline. For the surface sterilisation of chickpea (*Cicer arietinum* L.) seeds, a 10% (v/v) hypochlorite solution was used and washed several times with sterile distilled water. Further, the surface-sterilised seeds were soaked in the suspension containing 1.5 × 10$^8$ cells ml$^{-1}$ for 30 min for biopriming. About 15 untreated (control) and treated (bioprimed with *P. guariconensis* VDA8) seeds were placed in a sterile Petri dish assembly lined with moist filter paper. Seeds were incubated for seven days at 30°C. The experiment was conducted in triplicate to determine germination indices and plant growth as per Adebisi et al. (2010).

*Biocontrol activity.* Soil was procured from a nearby farm and intermittently sterilised (121°C, 20 min, 3 times) before experiment. Sick pots (n = 3), each containing 1.5 kg, were prepared by aseptically mixing sterile soil mycelial fragments of *F. oxysporum* (5%) before use. Further, the chickpea seeds (30 per pot) were sown in pots designated as: treatment I: non-chemoprimed or bioprimed (untreated) seeds + mycelial fragments of *F. oxysporum* (5%) in soil; treatment II: *P.guariconensis* VDA8 cell bioprimed seeds + mycelial fragments of *F. oxysporum* (5%) in soil as test; treatment III: non-chemoprimed or bioprimed (untreated) seeds as non-sick pot; and treatment IV: seeds chemoprimed with purified DAPG (30 µg ml$^{-1}$) + mycelial fragments of *F. oxysporum* (5%) in soil as test. Pots were watered intermittently for 15 days and uprooted before recording observations indicating biocontrol activity. Experiments were performed in triplicate for each treatment at 30°C. Parameters such as root length, shoot length, and germination percentage, coefficient of seed germination, seedling vigour index-I (SVI-I), seedling vigour index-II (SVI-II), and germination speed were calculated according to Adebisi et al. (2010).

*Insecticidal effects on treated grain.* The insecticidal effect of purified DAPG was assessed as per Hematpoor et al. (2017) on the streak of insect repellence, contact toxicity, and mortality assay.

For bioassay of insecticidal effects on treated grain, rice grains (5 g per test per experiment) were soaked either in 1 ml DAPG (0.5 mg in methanol; test) or only in methanol (as control) in Petri dish (9 × 1.5 cm) before drying in a fume hood. To each Petri dish, the post-harvest insect pests viz. *S. oryzae* or *R. dominica* adults (n = 10) were separately introduced before sealing. All treatments were monitored to record mortality as mean ± standard deviation (SD%).

For contact toxicity bioassay of DAPG, the residual film technique was used. A series of purified DAPG dilutions of 250, 500, and 1000 µg mL$^{-1}$ were prepared in methanol. Each dilution was used for soaking Whatman filter paper No. 1 placed in a Petri dish before drying in a fume hood at room temperature for 15 min. The control Petri dish with filter paper received only methanol. To each Petri assembly, adults of *S. oryzae* or *R. dominica* (ten of each) were introduced. All plates were sealed with parafilm and monitored for % mortality at 24 and 48 h of treatment, which was calculated using formula:

$$\% \text{ mortality} = \frac{(\% \text{ live in control} - \% \text{ live in treatment})}{\% \text{ live in control}} \times 100.$$

The repellence test of DAPG was performed using area preference method. Sterile circles of Whatman No. 1 filter paper were cut into halves and soaked with either DAPG solution (1 mg mL$^{-1}$) or methanol before drying in a fume hood (15 min, 25°C). Each half of DAPG and methanol-soaked filter paper was placed in a sterile Petri dish, and then either *S. oryzae* or *R. dominica* adults (n = 10 each) were introduced. The plates were sealed with parafilm, and the movement of the insects was periodically recorded for up to 20 h. The procedure was repeated four times and expressed as mean ± SD% of repellence calculated as:

$$\% \text{ of repellence} = \frac{(Nc - Nt)}{(Nc + Nt)} \times 100,$$

where Nc is the number of insects present on the control strip, Nt – on the treated strip.

# Results and discussion

***Screening of nutrient medium components by Plackett-Burman design (PBD).*** PBD is a useful statistical design to screen a large number of 'n' variables in just a smallest of 'n+1' number of trials. PBD is reported to screen the most significant factors that impact DAPG production and facilitates ignoring less significant factors. This statistical design also facilitates interaction-based selection of variables. Such short-listed significant variable(s) can be subjected to full-factorial design for the improvement of the fermentative process (Saharan et al., 2011). Hence, in the present study, 19 variables at high and low levels were coded in a 20-run PBD design to screen the most influential factors affecting fermentative DAPG production by *P. guariconensis* VDA8. Table 1 represents the PBD experimental plan for 20 trials at two levels of concentration for each variable along with the response, i.e., DAPG production (μg $ml^{-1}$). Further, the factorial design analysis revealed $p \leq 0.05$ for all the factors as shown in Tables 2 and 3 for ANOVA and regression analysis, respectively.

A significantly high *F*-value was recorded for aeration (32.74) and pH (26.28), followed by glycerol (15.4). From the regression model, the standardised effects of the analysed factors were estimated to screen the critical factors using the main effects plot and Pareto chart (Figures 1 and 2). Similarly, effects (8.356 and 7.486) and the coefficient (4.178 and 3.743) of aeration and initial pH indicated that a change in these variables would be more effective for DAPG production.

With respect to DAPG production, the main effects plot (Figure 1) indicated a negative effect of increasing aeration to 120 rpm; however, cell growth decreased as observed during OVAT analysis in the absence of aeration (unpublished data). The DAPG secondary metabolite might also be affected by the logarithmic growth phase and fermentation volume (a large volume requires more aeration for mass transfer and optimal metabolism). The present observation may have been realised due to a delay of the onset of the late log phase to favour biomass production rather than secondary metabolite (DAPG) production in addition to the small volume of the experimental setup (100 ml). Further, it was observed that raising pH towards alkaline (8 to 10) increased cellular stress to reduce production of DAPG. Among carbon sources, a higher concentration of glycerol (15 g $L^{-1}$) promoted DAPG production than sugars from sugarcane molasses.

***Table 2.*** ANOVA of DAPG produced by *Pseudomonas guariconensis* VDA8 during Plackett-Burman design (PBD) runs

| Source | df | Adj SS | Adj MS | *F*-value | *p*-value |
|---|---|---|---|---|---|
| Model | 11 | 1364.98 | 124.09 | 11.64 | 0.001 |
| Linear | 11 | 1364.98 | 124.09 | 11.64 | 0.001 |
| Glycerol | 1 | 164.23 | 164.23 | 15.40 | 0.004 |
| $NaH_2PO_4$ | 1 | 84.80 | 84.80 | 7.95 | 0.022 |
| $NH_4Cl$ | 1 | 88.43 | 88.43 | 8.29 | 0.021 |
| Molasses | 1 | 34.98 | 34.98 | 3.28 | 0.108 |
| $ZnSO_4 \times 7H_2O$ | 1 | 53.85 | 53.85 | 5.05 | 0.055 |
| $CoCl_2 \times 6H_2O$ | 1 | 76.66 | 76.66 | 7.19 | 0.028 |
| $CuCl_2 \times 2H_2O$ | 1 | 62.27 | 62.27 | 5.84 | 0.042 |
| $H_3BO_3$ | 1 | 116.67 | 116.67 | 10.94 | 0.011 |
| $NiCl_2 \times 6H_2O$ | 1 | 53.81 | 53.81 | 5.05 | 0.055 |
| Aeration, rpm | 1 | 349.11 | 349.11 | 32.74 | 0.000 |
| pH | 1 | 280.18 | 280.18 | 26.28 | 0.001 |
| Error | 8 | 85.30 | 10.66 | | |
| Total | 19 | 1450.28 | | | |

df – degree of freedom, SS – sum of squares; MS – mean square

***Table 3.*** Regression analysis of variables affecting DAPG production during Plackett-Burman design (PBD) runs

| Term | Effect | Coef | SE Coef | *T*-value | *p*-value |
|---|---|---|---|---|---|
| Constant | | 13.748 | 0.730 | 18.83 | 0.000 |
| Glycerol | 5.731 | 2.866 | 0.730 | 3.92 | 0.004 |
| $NaH_2PO_4$ | 4.118 | 2.059 | 0.730 | 2.82 | 0.022 |
| $NH_4Cl$ | 4.205 | 2.103 | 0.730 | 2.88 | 0.021 |
| Molasses | 2.645 | 1.322 | 0.730 | 1.81 | 0.108 |
| $ZnSO_4 \times 7H_2O$ | 3.282 | 1.641 | 0.730 | 2.25 | 0.055 |
| $CoCl_2 \times 6H_2O$ | −3.916 | −1.958 | 0.730 | −2.68 | 0.028 |
| $CuCl_2 \times 2H_2O$ | −3.529 | −1.764 | 0.730 | −2.42 | 0.042 |
| $H_3BO_3$ | −4.831 | −2.415 | 0.730 | −3.31 | 0.011 |
| $NiCl_2 \times 6H_2O$ | −3.281 | −1.640 | 0.730 | −2.25 | 0.055 |
| Aeration, rpm | 8.356 | 4.178 | 0.730 | 5.72 | 0.000 |
| pH | 7.486 | 3.743 | 0.730 | 5.13 | 0.001 |

Coef – regression coefficient, SE Coef – standard error coefficient

The findings of the positive role of glycerol as formulation stress protectant is evident in *Pseudomonas* biocontrol agents from previous studies (Craig et al., 2021). Sarma et al. (2010) optimised DAPG production to 20 mg $L^{-1}$ under batch cultivation when glycerol was 15 g $L^{-1}$ and C to N ratio was maintained at 12.5. Likewise, Bajpai et al. (2017) recorded high antibiotic secretion from a DAPG-producing *P. protegens* W45 strain at 2% v/v glycerol during media optimisation. Similarly, $NaH_2PO_4$ > NH4Cl > KCl and trace elements viz. $Zn^{+2}$ and $Ca^{+2}$ had a positive effect. In contrast, the presence of $H_3BO_3$ showed a negative effect. Similarly, a negative effect on DAPG production had trace elements viz. $Mg^{+2}$ (>0.04) and the presence of $Ni^{+2}$, $Co^{+2}$, and $Cu^{+2}$ ions; a negligible effect on DAPG production had the presence of nitrogen source urea and $Mn^{+2}$, and $MoO_4^{2-}$ ions. In a similar experiment for DAPG production from fluorescent *Pseudomonas*, only $Zn^{2+}$, $Mn^{2+}$, and $MoO_4^{2-}$ ions were found as the most significant, and the optimum values $Zn^{2+}$, $Mn^{2+}$, and $MoO_4^{2-}$ were 83, 42, and 135 M, respectively, to achieve 125 mg $L^{-1}$ of DAPG (Saharan et al., 2011). Eventually, the trace elements were kept to either low or removed from the optimised DAPG production.

Further, the Pareto chart analysis (Figure 2) indicated two out of 19 studied variables, i.e., aeration (rpm) and initial pH, to be the most influential to fermentative DAPG production from *P. guariconensis* VDA8. Thus, based on F, *p* and *t* values, aeration (rpm) and pH were used for CCD analysis.

***Optimisation of variables utilising central composite design (CCD).*** For superior optimisation of significant factors screened by PBD analysis, full-factorial design for using response surface methodology (RSM) was used. It is well known that CCD not just facilitates assessment of the most effective factors: the association among the variables can also describe the optimal conditions for maximum productivity. From the PBD analysis, it was interpreted that high aeration and alkaline pH negatively affects DAPG production. However, in the absence of aeration, bacterial growth would be drastically reduced and,

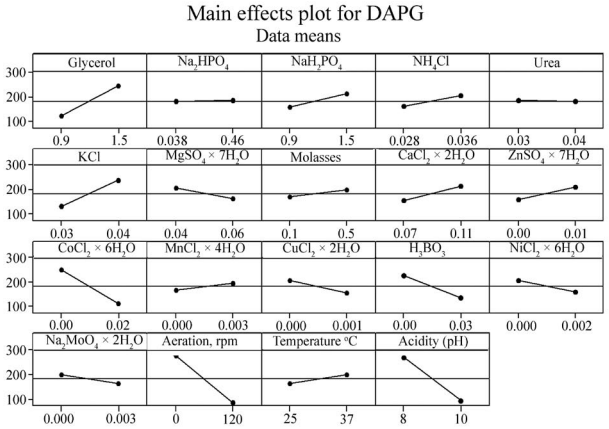



***Figure 1.*** Main effects plot of variables affecting DAPG production illustrating the effect of selected factors and response

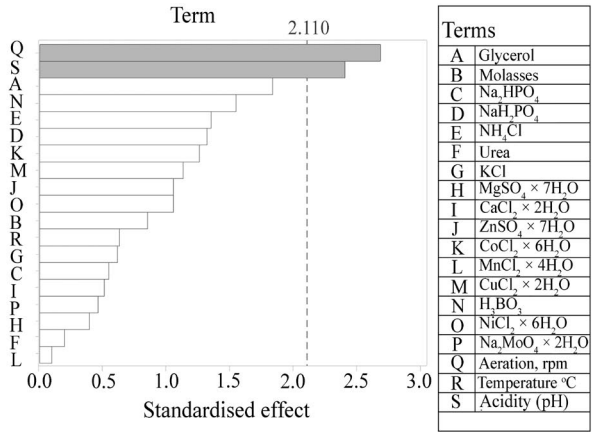



***Figure 2.*** Pareto chart analysis showing the order of the relative level of significance of variables for DAPG production by *Pseudomonas guariconensis* VDA8

therefore, a degree of aeration would be necessary to sustain the viability of fermentative cells. Statistical optimisation may also help to select the aeration rate suitable to support growth as well as production of DAPG in a large-volume continuous stirred tank reactor. Similarly, shifting pH to alkaline would increase metabolic stress and, therefore, it needs to be fine-tuned for maximal DAPG production. Consequently, CCD was used to determine the optimal level of the two variables, aeration (rpm) and pH for the DAPG production by *P. guariconensis* VDA8.

A total of 13 trial runs with different combinations of the two selected variables were performed. The modified optimal media components from the PBD analysis resulted in a higher DAPG yield than that of the conventional Schlegel's synthetic medium in the CCD matrix. Table 4 illustrates the actual variable levels with CCD experiments and the DAPG yield response. The values of regression (1) were found to be 5.35 and 0.024; model and model probability (2) were found to have *F*-value of 12.07 and $p < 0.05$, respectively, suggesting that the model was significant. The value of interaction and lack of fit probability were observed with $p > 0.05$ suggesting that they were insignificant (Table 5). A high *F* value of initial alkaline pH (13.48) and aeration (10.67 rpm) were indicated as most significant.

For the response surface model, the significance was checked by *F*-test and ANOVA. The value of lack of fit was insignificant ($p = 0.920$), and high correlation coefficient value ($R^2 = 87.82\%$) was observed. Accordingly, the second-order polynomial equation was found to adequately represent DAPG production as DAPG (μg ml$^{-1}$) = 684 + 6.12 and aeration (rpm) − 46.6 pH.

The three-dimensional response surface plot and the contour plot showed a curvature along the aeration (rpm) and pH (Figures 3 and 4) and represented the interaction of the two variables. The response optimiser algorithm suggested that with high (144 rpm) aeration and pH of 8.92 maximal DAPG production of 1404.17 μg ml$^{-1}$ could be achieved. This may be due to the significance of the quadratic coefficients of these variables in the model.

To validate the statistical model prediction, DAPG was produced in large volume flasks (5 L) with modified Schlegel's medium (1 L) in duplicate. Sterile medium was inoculated with 24 h old culture of *P. guariconensis* VDA8 at a cell concentration equivalent to an optical density of 0.5. Incubation was carried out in a rotary shaker incubator at 144 rpm and 30°C for 72 h. Further extraction, purification, and quantification by HPLC resulted in a DAPG yield of 1.26 ± 0.68 g L$^{-1}$, a yield intermediate to the

***Table 4.*** Central composite design (CCD) matrix with experimental response of DAPG production by *Pseudomonas guariconensis*

| Run | Point type | Block | Aeration, rpm | pH | DAPG μg ml$^{-1}$ |
|---|---|---|---|---|---|
| 1. | 1 | 1 | 0 | 8 | 306.45 ± 0.07 |
| 2. | 1 | 1 | 120 | 10 | 683.00 ± 0.60 |
| 3. | 0 | 1 | 60 | 9 | 689.09 ± 0.86 |
| 4. | 0 | 1 | 60 | 9 | 686.14 ± 0.37 |
| 5. | 0 | 1 | 60 | 9 | 682.86 ± 0.41 |
| 6. | −1 | 1 | 60 | 10.41421 | 389.05 ± 0.92 |
| 7. | 1 | 1 | 120 | 8 | 798.55 ± 0.65 |
| 8. | 1 | 1 | 0 | 10 | 137.84 ± 0.83 |
| 9. | −1 | 1 | 60 | 7.585786 | 451.62 ± 0.58 |
| 10. | −1 | 1 | 144.8528 | 9 | 1669.50 ± 0.71 |
| 11. | 0 | 1 | 60 | 9 | 694.86 ± 0.76 |
| 12. | 0 | 1 | 60 | 9 | 697.97 ± 0.88 |
| 13. | −1 | 1 | −24.8528 | 9 | 325.76 ± 0.51 |

*Note.* Response values of DAPG shown as mean ± SD of experiments conducted in triplicate.

***Table 5.*** Central composite design (CCD) for optimisation of nutrient medium components for DAPG production by *Pseudomonas guariconensis*

| Source | df | Adj SS | Adj MS | *F* | *P* |
|---|---|---|---|---|---|
| Regression | 5 | 912265 | 182453 | 5.35 | 0.024 |
| Linear | 2 | 823519 | 411759 | 12.07 | 0.005 |
| Aeration, rpm | 1 | 363975 | 363975 | 10.67 | 0.014 |
| pH | 1 | 459544 | 459544 | 13.48 | 0.008 |
| Square | 2 | 73312 | 36656 | 1.07 | 0.392 |
| rpm × rpm | 1 | 1520 | 1520 | 0.04 | 0.0839 |
| pH × pH | 1 | 67895 | 67895 | 1.99 | 0.201 |
| Interaction | 1 | 15434 | 15434 | 0.45 | 0.523 |
| rpm × pH | 1 | 15434 | 15434 | 0.45 | 0.523 |
| Residual error | 7 | 238707 | 34101 | | |
| Lack-of-fit | 3 | 25228 | 8409 | 0.16 | 0.920 |
| Pure error | 4 | 213479 | 53370 | | |
| Total | 12 | 1150972 | | | |

df – degree of freedom, SS – sum of squares, MS – mean square; * – significant at $p < 0.05$, $R^2 = 87.82\%$, $R^2$ (adjusted) = 79.12%

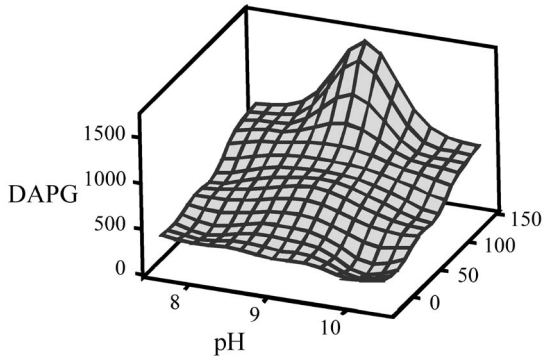



***Figure 3.*** Response surface graph for DAPG production by *Pseudomonas guariconensis* VDA8 showing the interaction of pH and aeration (rpm)

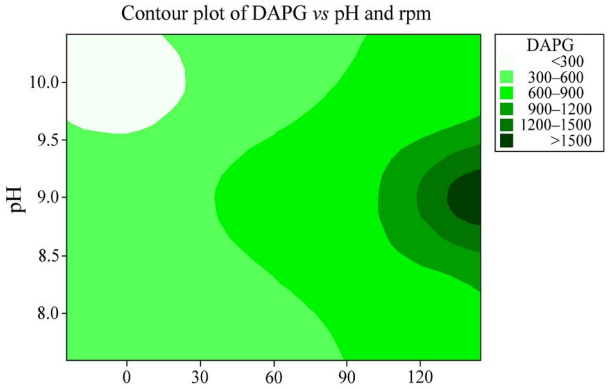



***Figure 4.*** Contour plots for DAPG production by *Pseudomonas guariconensis* VDA8 showing the interaction of two variables, pH and aeration (rpm)

CCD-predicted 1.4 g $L^{-1}$ and 1.66 ± 0.71 g $L^{-1}$ DAPG achieved during the shake flask experiment (performed at 100 ml volume) for evaluating CCD. In a similar attempt, a study conducted in a 14 L bioreactor resulted in 0.135 g $L^{-1}$ of DAPG at the end of 36 h of cultivation by fluorescent Pseudomonad R62 (Saharan et al., 2011). High initial cell concentration may also favour quorum sensing and induce secondary metabolite production (Kankariya et al., 2019).

The experiments of the present study suggest that DAPG production from *P. guariconensis* VDA8 is also largely affected by the initial cell concentration and fermentation volume (probably functions as a significant covariate). Thus, to suit pilot and industrial-scale production volumes, there is significant scope to include these variables for further statistical optimisation studies. To our current knowledge, the highest DAPG production of 1.2 g $L^{-1}$ by *P. fluorescens* S272 with a heat shock in complex medium and 1.28 g $L^{-1}$ yield has been achieved by metabolically engineered *Escherichia coli* (Nakata et al., 1999; Zha et al., 2009). Thus, from the present study, *P. guariconensis* VDA8 appears as the most suitable candidate for industrial scale production of DAPG.

***Characterisation of purified DAPG.*** A nearly identical UV-spectral profile with a major peak ($\lambda_{max}$) at 270 nm and a minor peak at 330 nm of DAPG as compared to reference DAPG was observed. The FTIR analysis indicated that the purified DAPG generated similar signals corresponding to the reference DAPG. It showed the presence of different bands corresponding to the following functional groups as: (1) broad absorption from 3600 to 3200 $cm^{-1}$ corresponding to a bounded hydroxide (OH) stretching; (2) bands from 2970 to 1850 $cm^{-1}$ due to the stretching of $CH_3$ group attached to a benzene ring presented the band absorption from 1630 $cm^{-1}$ to 1430 $cm^{-1}$; (3) the stretching of the carbonyl group from 1700 to 1680 $cm^{-1}$; and (4) band from 1100 to 130 $cm^{-1}$ due to alcohols, ethers and acid esters. The values represent functional groups in agreement to the reference DAPG and earlier analysis (Ayyadurai et al., 2006). The TLC analysis of DAPG showed single fluorescent spot under UV light with identical $R_f$ values of 0.35 corresponding to reference DAPG. The reach results agree with the $R_f$ = 0.35, as reported by Reddy et al. (2007). The HPLC chromatogram of purified DAPG displayed a single peak at retention time of 8.1 min. The HPLC was run for 10 min, which ensured adequate resolution of DAPG peak and quicker elution time. Sharp isolated peaks at typical absorbance at 270 and 330 nm indicated purity of the methanol extract and agree with previous report (Shanahan et al., 1992). The electrospray ionization-quadrupole-time of flight-mass spectrometry (LC ESI-MS Q-TOF) analysis corresponding to DAPG was confirmed by the intense stable base peak at m/z 211.02 (M + H), while shoulder peaks were observed at m/z 211.76 and 212.06 (M + H). The mass spectrum was largely identical to reference DAPG, which confirmed compound composition as $C_{10}H_{10}O_5$ (Figure 5). The results are in accordance with Brucker et al. (2008) and Zhou et al. (2012).

***Chickpea seed germination assay.*** Chickpea is a legume pulse crop, and the seeds are a good cholesterol-free nutritional source rich in carbohydrates, proteins, vitamins, and minerals (Gowda et al., 2018). Its global annual cultivation exceeds 14.81 million ha (>20% among pulse crops) with a total production of 15.08 million tonnes in 2020. India is the world's largest producer of chickpeas with 6.94 million tonnes produced in 2021 (FAO, 2021b). The plants are sensitive to climate change and susceptible to over 50 diseases accounting to about 10% annual loss. Most of the loss is induced by *Fusarium* wilt, root, and collar rots caused by *F. oxysporum* and related species (Sarmah et al., 2012; Vandana et al., 2020). In the present study, combined biocontrol and PGP effect of *P. guariconensis* VDA8 on chickpea were studied by plate and later by pot assay.

The plate assay was conducted specifically to assess PGP activity of the DAPG-producing *P. guariconensis* VDA8 by seed biopriming. The bacterised seeds were incubated for seven days at 30°C, which showed higher germination rate (%) in treated than the untreated control (Figure 6).

It was observed that 93.3% of bioprimed seeds germinated against 73.3% germination in control, and biopriming improved the seed vigour index to 4.0 ± 0.11 as compared to 1.67 ± 0.0 in untreated control. Germination test is accepted as an index of seed quality and can be extended to assess the PGP effect of microbes (Gowda et al., 2018). In the present study, the PGP activity of *P. guariconensis* VDA8 was evident from the experiment owing to high seed vigour index than control.

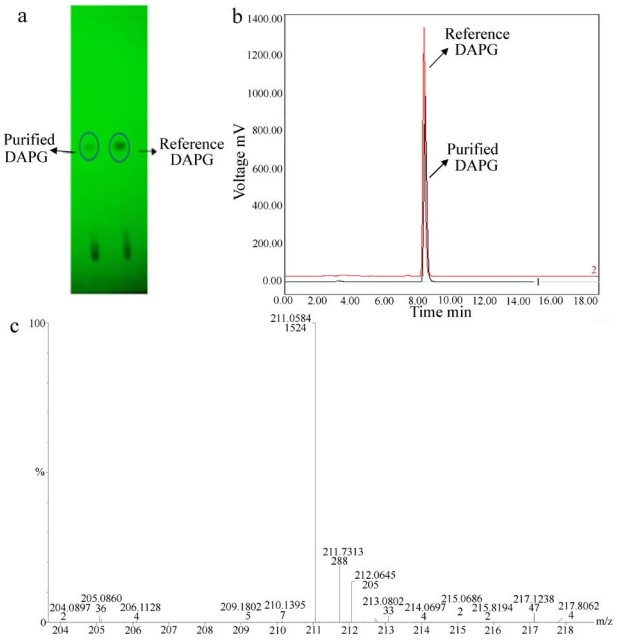
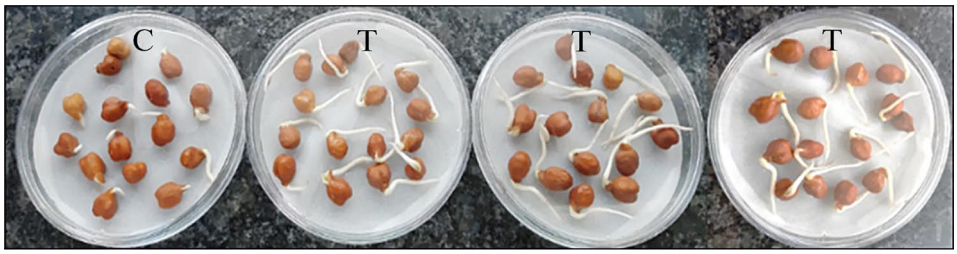



***Figure 5.*** Thin layer chromatography (TLC) (a), high-performance liquid chromatography (HPLC) (b), and electrospray ionization-quadrupole-time of flight-mass spectrometry (LC ESI-MS Q-TOF) (c) analysis of purified DAPG obtained from *Pseudomonas guariconensis* VDA8




Labels: C – non-primed/untreated (control), T – bioprimed (test)

***Figure 6.*** Biopriming plant-growth promoting (PGP) effect of *Pseudomonas guariconensis* VDA8 on chickpea seed germination shown after seven-day incubation at 30°C

***Biocontrol activity*** of *P. guariconensis* VDA8 and purified DAPG were assessed by pot assay against the fungal phytopathogen *F. oxysporum.* The results are summarised in Table 6 and depicted in Figure 7.

The sick pot control (treatment I) showed lower germination indices and stunted growth. The comparatively low germination percentage (77%), germination index (10.58), coefficient of seed germination (1.56), SVI-I and SVI-II (7.13 and 0.18), and smallest leaf length indicated a disease manifestation that severely affected plant growth. In contrast, the non-primed and non-pathogen-challenged pot (treatment III) in all respects showed better germination, seedling growth indices, and plant growth as compared to sick pot (treatment I). Among the biocontrol pots, the DAPG chemoprimed seeds (treatment IV) showed comparatively higher germination rate than *Fusarium*-challenged sick pot control (treatment I) or non-challenged control (treatment III). As compared to the sick pot control, the germination percentage improved to 92.2% from 77.78%, while SVI-I and SVI-II increased to 20.84 and 0.52, respectively, against corresponding meagre values of 7.13 and 0.18. Accordingly, there was obvious restriction of *Fusarium* infection and disease control due to DAPG chemopriming.

A comparison of all the treatments showed that the best outcome was recorded in the pot of bioprimed seeds challenged with *Fusarium* mycelia (treatment II). The highest germination percentage (95.5), germination speed (2.76), coefficient of seed germination (1.91), root length (8.3 cm), plant height (23.7 cm), and leaf length (0.6 cm) were recorded. As compared with the DAPG chemoprimed pot (treatment IV), a better outcome was observed for all indices indicating that the *P. guariconensis* VDA8 has an obvious PGP effect in addition to *Fusarium* biocontrol benefit, most probably via DAPG production.

PGP effects of *P. guariconensis* VDA8 can be attributed to production of indole acetic acid (IAA), siderophore, exopolysaccharide, cellobiase, pectinase, and tannase. The strain solubilises phosphate, secretes ammonia and is capable of growth on nitrogen-source devoid Ashby's growth medium, which is indicative of atmospheric dinitrogen fixation (unpublished data). These metabolic characteristics are largely similar to other closely related DAPG-producing fluorescent Pseudomonads, which induce systemic resistance in plants and contribute to disease suppression in soils (Haas, Défago, 2005).

***Table 6.*** Treatments and growth indices observed for *Fusarium* biocontrol activity in chickpea seeds imparted by biopriming of *Pseudomonas guariconensis* VDA8 and purified DAPG

| Treatment | I | II | III | IV |
|---|---|---|---|---|
| *Fusarium oxysporum* mycelia in soil | + | + | − | + |
| *P. guariconensis* VDA8 priming | − | + | − | − |
| DAPG (30 μg ml$^{-1}$) chemopriming | − | − | − | + |
| Germination percentage % | 77.78 ± 0.57 | 95.56 ± 1.5 | 91.11 ± 1.8 | 92.22 ± 0.5 |
| Germination speed | 2.66 ± 0.81 | 2.76 ± 0.52 | 2.67 ± 0.85 | 2.69 ± 0.72 |
| Germination index | 10.58 ± 0.84 | 12.08 ± 0.51 | 12.50 ± 0.74 | 11.90 ± 0.52 |
| Coefficient of seed germination | 1.56 ± 0.03 | 1.91 ± 0.05 | 1.87 ± 0.06 | 1.89 ± 0.01 |
| Shoot length cm | 6.73 ± 1.04 | 15.33 ± 0.08 | 15.30 ± 1.05 | 15.67 ± 1.0 |
| Root length cm | 2.43 ± 0.20 | 8.37 ± 0.32 | 7.30 ± 0.45 | 7.50 ± 0.35 |
| Plant height cm | 11.83 ± 0.40 | 23.70 ± 1.25 | 22.60 ± 1.60 | 23.17 ± 1.35 |
| Seedling vigour index I (SVI-I) | 7.13 ± 1.92 | 22.65 ± 2.10 | 20.59 ± 2.03 | 20.84 ± 2.0 |
| Seedling vigour index II (SVI-II) | 0.18 ± 0.02 | 0.51 ± 0.01 | 0.44 ± 0.02 | 0.52 ± 0.01 |
| Leaf length cm | 0.33 ± 0.05 | 0.67 ± 0.03 | 0.57 ± 0.03 | 0.60 ± 0.04 |

*Note.* Values shown as mean ± SD of experiments conducted in triplicate; + – present, − – absent.


*Note.* Treatments (I, II, III, and IV) photographed after 15 days of incubation at 30°C.

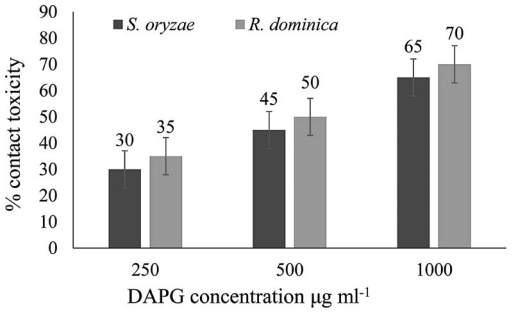
***Figure 7.*** *Fusarium* biocontrol activity of *Pseudomonas guariconensis* VDA8 and DAPG examined by biopriming and chemopriming in chickpea pot assay

The results are also in accordance with Saikia et al. (2009), who recorded phosphate solubilisation and siderophore production as a PGP mechanism by *P. fluorescens* to *Fusarium* wilt of chickpea. Similarly, Dey et al. (2004) attributed IAA and siderophore production by *P. fluorescens* for indirect stimulation of plant growth by improving soil pH and preventing the growth of certain fungi. By altering its architecture, phytopathogen abatement by PGP (capable to produce IAA and ammonia) promotes water captivation for faster seed germination and leads to greater root holding capacity (Haas, Défago, 2005). Gupta and Gopal (2008) attributed antagonistic activities of several PGP bacteria including *P. fluorescens* to the competitive advantage of siderophores to chelate iron and other heavy metals required for fungal growth. In the present study, *P. guariconensis* promoted root and shoot growth, suppressed fungal growth and disease occurrence, reduced the number of seedlings with wilting symptoms, increased plant growth parameters in agreement to previous reports. Overall, *P. guariconensis* VDA8 biopriming showed a convincing positive influence on plant growth and *Fusarium* wilt disease suppression.

***Insecticidal effects on treated grain.*** Insect pests-mediated biotic stress affect one-fifth of the crop produce amounting to ~US $470 billion. There are about 10,000 insect

species recognised to damage crops, among which ~10% are major threats (Sharma et al., 2017). For grain protection, chemical insecticides containing fumigants are used to prevent insect attacks during storage. Although the demand and use of insecticides have remained roughly the same in the last decade, there is (1) extensive development of insecticidal resistance, (2) environmental impact due to repetitive and stupendous use of chemical insecticides, and (3) increasing concerns on human and animal health. Consequently, the use of numerous chemical pesticides has been prohibited for grain protection over the last three decades. Alternatively, interest is increasing in naturally occurring microbial antagonistic compounds, which are biodegradable and amenable to industrial fermentative production. The main culprits in post-harvest storage loss of grains viz. larvae of *S. oryzae* (L.) and *R. dominica* (F.) are internal feeder, while their adults ingest intact grain (Hematpoor et al., 2017). In the present study, insecticidal activity of purified DAPG was evaluated against these two insect pests in the light of (1) mortality bioassay, (2) contact toxicity, and (3) repellence test. The toxicity potential and repellence of purified DAPG (1 mg ml$^{-1}$) against adults of *S. oryzae* and *R. dominica* are presented in Table 7, while DAPG contact toxicity is illustrated in Figure 8.

Based on the results obtained, purified DAPG demonstrated repellency to both insects within 5 h and continued up to 20 h with more repellence to *R. dominica* than *S. oryzae* adults. The insecticidal efficacy of purified DAPG




*Note.* Error bars represent ± SD of independent duplicate experiments.

***Figure 8.*** Percentage (%) of contact toxicity of DAPG against the adults of *Sitophilus oryzae* and *Rhyzopertha dominica* at 48 h

***Table 7.*** Mortality and repellence activity of purified DAPG (1 mg ml$^{-1}$) against adults of *Sitophilus oryzae* and *Rhyzopertha dominica*

| | Repellency activity | | | | Mortality % | |
|---|---|---|---|---|---|---|
| Insects | 5 h | 10 h | 15 h | 20 h | 72 h | control |
| *S. oryzae* | 29.00 ± 5.65 | 37.92 ± 6.90 | 42.50 ± 3.50 | 43.92 ± 1.50 | 55.00 ± 1.40 | 0.00 ± 0.00 |
| *R. dominica* | 33.66 ± 4.70 | 43.92 ± 1.50 | 46.42 ± 5.50 | 52.77 ± 3.90 | 65.00 ± 0.70 | 0.00 ± 0.00 |

*Note.* Data represents the mean ± SD of triplicate experiment.

at concentration of 1 mg ml$^{-1}$ remained relatively weak up to 48 h; however, toxicity progressively increased to 55% and 65% against *S. oryzae* and *R. dominica,* respectively, possibly due to initial repellency and delayed gut absorption. It can be postulated that after the consumption of treated rice grains until 72 h evidenced insecticidal activity of DAPG at the selected concentration.

The contact toxicity bioassay demonstrates the effect of varying concentrations of purified DAPG in the absence of rice grains on soaked filter paper. Interestingly, mortality against both insects was observed to be high as compared to the treated rice grain bioassay and contact toxicity was exerted only after 24 h. After 48 h, mortality was observed in *R. dominica* rather than *S. oryzae* adults even at low DAPG concentrations of 250 µg ml$^{-1}$ (35% and 30%, respectively). Contact toxicity proportionately increased for *R. dominica* as well as *S. oryzae* adults with increase in DAPG concentration to 500 µg ml$^{-1}$ (50% and 45%, respectively) and finally reached highest (65% and 70%, respectively) at 1000 µg ml$^{-1}$ DAPG. Higher mortality in the contact toxicity assay than in rice grain bioassay was probably due to the absence of grain feed. It coincides with the results of Hematpoor et al. (2017) that reported the effect of three bioactive compounds obtained from plant extracts.

There exists certain rhizobacterial *Pseudomonas* spp. that exhibit insect pathogenicity as an additional trait to biocontrol activity against phytopathogens (Kupferschmied et al., 2013). Péchy-Tarr et al. (2008) identified a novel gene cluster 'makes caterpillars floppy' encoding an insect toxin Mcf1 in DAPG-producing *P. fluorescens*. Yet there are only few reports that demonstrate the insecticidal effect of fluorescent Pseudomonads, but only *P. protegens* and *P. chlororaphis*, owing to the presence of *fit* genes (*P. f luorescens* insecticida l toxin) that encode proteinaceous toxin (Flury et al., 2016). However, there are no substantial evidence that pinpoint DAPG as a bioactive compound for contact toxicity, especially towards *R. dominica* and *S. oryzae.* Lu et al. (2010) genetically engineered a DAPG-producing *P. fluorescens* to express *Androctonus australis* Hector insect toxin 1 (AaHIT1), the scorpion neurotoxin peptide to impart insecticidal activity. The wild-type and engineered bacterial strain retained antagonism against a range of phytopathogens owing to DAPG production and demonstrated to be insecticidal against *Mythimna seperata*. Flury et al. (2017) conducted gene deletion assays and identified cyclic lipopeptides and HCN produced by PGP *Pseudomonas* strains *P. protegens* CHA0, *P. chlororaphis* PCL1391, and *Pseudomonas* sp. CMR12a as insecticides against the larvae of *Galleria mellonella* and *Plutella xylostella*. The authors suspected the possible insecticidal role of DAPG, but the present study demonstrates direct evidence of its insecticidal activity.

Overall, the findings prove that DAPG is also a potential natural insecticide candidate for control of adult insect pests as it is biodegradable and does not show toxicity to higher animals (Dhanasekaran, Thangaraj, 2014). Toxicity-based evidence in higher organisms would be needed to further evaluate the concentration and time, along with assays, to ascertain biosafety before deploying fermentative DAPG for commercial and domestic applications.

# Conclusions

1. Aeration (rpm) and alkaline pH are the crucial factors that affect DAPG production by *Pseudomonas guariconensis* VDA8. Besides initial cell concentration, media and reactor volume can profoundly affect cell growth and secondary metabolism for DAPG production in large-volume fermentation during scale-up. Even then a high yield of 1.26 g L$^{-1}$ DAPG can be achieved. Components of modified Schlegel's synthetic medium can be amended or replaced with cheaper alternatives for more economical industry-scale DAPG production.

2. *P. guariconensis* VDA8 emerged as a dual plant-growth promoting (PGP) and biocontrol alternative for *Fusarium* phytopathogen suppression in chickpea and can be extended to other crops even in moderately saline and alkaline soil.

3. With 55–60% mortality, DAPG produced by *P. guariconensis* VDA8 has effective insecticidal activity against post-harvest insect pests *Sitophilus oryzae* and *Rhyzopertha dominica* adults.

4. The newly discovered *P. guariconensis* VDA8 strain is a valuable arsenal and cell factory for biotechnological application and most suitable PGP candidate for sustainable agriculture.

## Acknowledgements

The research was supported by departmental grants of University Grants Commission, New Delhi (F.4-23/2015/DRS-III(SAP-II)), Department of Science and Technology, New Delhi (SR/FST/LS-II/2017/101(C)) and (SR/WOS-A/LS-1209/2014(G)), and Rajiv Gandhi Science and Technology Commission, Mumbai (KBCNMU/RGSTC/SanctionOrder/30(LSR-13).

Received 02 10 2021
Accepted 04 03 2022

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# Naujai atrasto vidutiniškai haloalkalitolerantiško *Pseudomonas guariconensis* VDA8 gaminamo 2,4-diacetylphloroglucinolio statistinis gamybos optimizavimas, *Fusarium* biokontrolė ir insekticidinis aktyvumas

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## Santrauka

Pastebėta, kad naujai atrasta rizobakterijų padermė *Pseudomonas guariconensis* VDA8 išskiria antimikrobinę medžiagą 2,4-diacetilflorogliucinolį (DAPG). Padermė pasižymi augalų augimą skatinančiomis savybėmis: indolo acto rūgšties, sideroforų, egzopolisacharidų, amoniako ir ličio fermentų gamyba, fosfatų tirpinimu; be to, ji toleruoja druskas (0,85 M) ir šarmus (pH 8,5). Auginimo sąlygų optimizavimas maksimaliai DAPG produkcijai pasiekti buvo ištirtas taikant statistinės analizės priemones Plackett-Burmano dizainą (PBD) ir centrinį sudėtinį dizainą (CCD). Su PBD atlikus 20 testų nustatyta, kad iš 19 Šlegelio sintetinės terpės sudedamųjų maistinių dalių ir fizikocheminių veiksnių didžiausią įtaką turi aeracija ir pH. Siekiant parodyti, kad 144 apsisukimų per minutę aeracijos greitis ir terpės pH 8,9 sudaro optimalias sąlygas 14 g $L^{-1}$ DAPG gamybai purtomoje kolboje, visų įmanomų kombinacijų penkių lygių CCD atlikta naudojant 13-os eksperimentų modifikuotoje Šlegelio terpėje duomenis. CCD modeliui patvirtinti atlikus 1 l tūrio fermentaciją, gauta 1, 26 g $L^{-1}$ išeiga ir nustatyta, kad *P. guariconensis* gaminamam DAPG svarbūs veiksniai taip pat yra terpės tūris ir pradinis ląstelių tankis. Be to, lyginant su neapdorotomis sėklomis, sėjamojo avinžirnio (*Cicer arietinum* L.) sėklų biologinis apdorojimas jų pagerino daigumą (93,3 %) ir gyvybingumo indeksą (4,0). Vazonuose atliktas *Fusarium oxysporum* fitopatogenų biokontrolės aktyvumo testas parodė, kad 30 μg $ml^{-1}$ DAPG apdorotos sėklos pasižymėjo didžiausiu daigumu (95,5 %), daigų gyvybingumu ir augalų augimo rodikliais, lyginant su neapdorotomis, ir įrodė, kad *P. guariconensis* VDA8 pasižymi kombinuotu augalų augimo skatinimo ir biokontrolės aktyvumu. Be to, išgrynintas DAPG (1 mg $ml^{-1}$) pasižymėjo repelentiniu ir insekticidiniu poveikiu prieš maisto produktų sandėliavimo kenkėjus *Sitophilus oryzae* ir *Rhyzopertha dominica* suaugėlius: po 72 val. chemiškai apdorotuose ryžiuose jų mirtingumas siekė 55–60 %. Nustatyta, kad esant panašiai koncentracijai tiesioginio kontakto toksiškumas per 48 val. pasiekia 66–70 %. Eksperimento duomenys patvirtina, kad *P. guariconensis* VDA8 galima naudoti kaip dvigubą augalų augimo skatinimo ir biokontrolės agentą druskingame bei šarminiame dirvožemiuose, o išgrynintą DAPG – kaip stiprų insekticidą grūdus saugant po derliaus nuėmimo.

Reikšminiai žodžiai: Plackett-Burmano dizainas, centrinis sudėtinis dizainas, *Cicer arietinum*, *Fusarium oxysporum*, *Sitophilus oryzae*, *Rhyzopertha dominica*.