FORMULATION OF *PSEUDOMONAS FLUORESCENS* AS A BIOPESTICIDE AGAINST SOIL BORNE ROOT PATHOGENS

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ABSTRACT

***Pseudomonas fluorescens* is one of the most beneficial rhizosphere bacteria mostly used to control broad range of soil borne plant pathogens. The local isolate of *P.fluorescens* (PFDS) was formulated using calcium carbonate ($CaCo_3$), starch, grind rice and grind hornwort (*Ceratophylum demersum L.*) as carriers to examine the best carrier for application of bacteria to control the plant pathogenic fungi *Fusarium solani* and *Rhizoctonia solani* and root-knot nematode *Meloidogyne javanica*. The results revealed a significant superiority of starch and $CaCo_3$ (35.3, 32.3 $\times 10^7$ CFU ml^{-1}) respectively compare to the other carriers with no significant differences among them, with nearly similar pattern of viability along six months of storage at room temperature (8.66, 8, 6.66, 4.33, 4.33 and 2 $\times 10^9$ CFU ml^{-1}) and (4.66, 4.66, 5, 4.33, 3.66 and 2.33 $\times 10^9$ CFU ml^{-1}) respectively. The monthly test showed that $CaCo_3$- *P.fluorescens* formulation was represented high inhibition *in vitro* to the radial growth of *F.solani* (81.48, 79.26, 80.66, 80.31, 79.2 and 55.55 %) respectively and *R.solani* (74.07, 73.33, 72.22, 70.37, 66.66, and 44.44%) respectively along six months of storage with significant differences among treatments of each group. $CaCo_3$ formulation at a concentration of 5 gm l^{-1} reduced in *vitro* eggs hatching to 5% and 44% death of the 2nd stage juvenile of *M.javanica*.**

Keywords: *Pseudomonas fluorescens*, Biopesticides formulation, *Fusarium solani, Rhizoctonia solani, Meloidogyne javanica*

تحضير مستحضر حيوي للبكتريا *Pseudomonas fluorescens* لمقاومة مسببات امراض الجذور الموجودة في التربة

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المستخلص

تعد البكتريا ***Pseudomonas fluorescens*** واحدة من أكثر أنواع بكتريا التربة المفيدة التي تستخدم على نطاق واسع في مقاومة مسببات امراض النبات التي تنتقل عن طريق التربة. تضمنت الدراسة اختبار كفاءة اربعة مواد حاملة وهي كاربونات الكالسيوم ($CaCo_3$) والنشأ ودقيق الرز ومسحوق نبات الشمبلان (***Ceratophylum demersum L.***) كل على حدة. لتجهيز العزلة المحلية من بكتريا ***P.fluorescens*** (PFDS) كمستحضر حيوي ضد المسببات المرضية الفطرية ***Fusarium solani*** و ***Rhizoctonia solani*** ونيماتودا العقد الجذرية ***Meloidogyne javanica***. أوضحت النتائج تفوق كل من النشأ وكاربونات الكالسيوم معنويا على باقي المواد الحاملة (35.3 و32.3 × 10^7 وحدة تكوين مستعمرة $مل^{-1}$) على التوالي وبدون فروق معنوية بينهما مع سلوك مشابه بالنسبة لحيوية البكتريا خلال ستة اشهر من الخزن في درجة حرارة الغرفة (8.66 و8 و6.66 و4.33 و4.33 و2 × 10^9 وحدة تكوين مستعمرة $مل^{-1}$) و(4.66 و4.66 و5 و4.33 و3.66 و2.33 × 10^9 وحدة تكوين مستعمرة $مل^{-1}$) على التوالي. أظهر الاختبار الشهري لكفاءة المستحضر الحيوي $CaCo_3$–***P.fluorescens*** ولمدة ستة اشهر تثبيطا عاليا للنمو القطري للفطر ***F.solani*** (81.48 و79.26 و80.66 و80.31 و79.22 و55.55) على التوالي وللفطر ***R.solani*** (74.07 و73.33 و72.22 و70.37 و66.66 و44.44) على التوالي وبفروقات معنوية بين المعاملات لكل مجموعة على حدة. أدى استخدام المستحضر الحيوي $CaCo_3$– ***P.fluorescens*** مختبريا بتركيز 5غم $لترا^{-1}$ إلى خفض معدل فقس بيوض النيماتودا ***M.javanica*** إلى 5% ومعدل قتل ليرقات الطور الثاني بلغ 44%.

كلمات مفتاحية: *Pseudomonas fluorescens*، *Fusarium solani*، *Rhizoctonia solani*، *Meloidogyne javanica*،

***Received:12/8/2017, Accepted:26/12/2017**

INTRODUCTION

Plant diseases and nutrition status playing a vital role in plant growth which is lead to reduce the quality and/or quantity of plant production. The classical and fast solution for these problems in agriculture mostly restricted in the use of chemical fertilizers and pesticides to avoid losses and increase yield regardless of the huge threat to the health and environment (25). According to the wide development in plant production especially in the biotechnology field, bio-resources effectively employed to replace the hazardous chemical materials to achieve environmentally friendly, long term and high throughput crop production processes (24). One of the most effective solutions to the mentioned problems represented by using of bacterium *Pseudomonas fluorescens* which is well-known as plant growth promoter and plant protectant against a wide range of plant pathogens in the rhizosphere area (24, 25). The application of *P.fluorescens* to the soil always considered as challenge to the scientists according to the complex chemical, physical and biological condition of soil environment which is make it of difficult to keep bacteria in best conditions of viability and activity as long as possible (15, 20, 27). The formulation of the bio-agent mostly forming an important factor influencing bio-pesticides activity and determine the role of application with high performance under field conditions (15, 22). The formulation process mostly involved a composition bio-agents with a suitable carrier and/or additives that guaranteed ease of delivery, long storage and economically inexpensive (7). Litterateur reviewed several types of organic and inorganic carriers used to formulate *P.fluorescens* such as starch, peat, compost, talk, clay-based carriers, manure, alginate, $CaCo_3$ *etc*. (3, 7, 19, 28). The objective of this study was to evaluate the availability of considering some organic and inorganic materials as carriers to formulate *P.fluorescens* for application as biopesticide against some soil borne plant pathogens.

MATERIALS AND METHODS

Microorganisms: The fungal isolates, *Fusarium solani* and *Rhizoctonia solani* were isolated locally from infected eggplants roots with root rot and root knot diseases. The isolates were purified and identified according to Parmeter and Whitney (17) and Booth (5). The Nematodes *Meloidogyne javanica* were directly isolated of Eggplant root knots and identified according to Luc *et al.* (13). *Pseudomonas fluorescens* DS (PFDS) isolate was provided by Dr. Dhya Al-Waily, Dept. of Plant Protection, College of Agriculture, Univ. of Basrah. *Pseudomonas fluorescens* powder formulations: The bacterium *P. fluorescens* was grown on King Broth medium (K.B.) at 28˚C for 24h (9). Calcium carbonate, starch, ground rice, and hornwort (*Ceratophylum demersum L.*) powder were separately proposed to be carriers to formulate *P. fluorescens* DS as powder formulation. 1 kg of each carrier autoclaved in a double at 121˚C and 15 pound/inch^2 for 30 min then mixed well with 400 ml of one day old *P. fluorescens* (306.7×10^{11} CFU ml^{-1}) under sterile conditions. The mixtures were packed in polyethylene bags with a moisture content 35% and stored in laboratory temperature until the end of experiments (28). *Pseudomonas fluorescens* viability in powder formulations: The viability of *P. fluorescens* in starch calcium carbonate powder formulations was examined monthly using dilution method (1 gm of each powder was diluted in 9ml of sterilized distilled water then serial of dilutions (10^{-1}- 10^{-9}) were prepared). K.B.A. 9cm plates were inoculated with 1 ml of $10^{-7,}$ 10^{-8} and 10^{-9} dilutions in four replicates. The plates incubated at 28˚C for 24h then the bacterial population was determined depending on colony counting to estimate colony formed unit CFU ml^{-1}. *Pseudomonas fluorescens* bioactivity against fungi and nematodes:= Serial dilutions 0, 0.1%, 0.2%, 0.3%, 0.4%, 0.5% w/v of *P.fluorescens* calcium carbonate powdered formulation. One ml of each dilution was mixed with 20 ml of P.D.A. (Potato dextrose agar (Oxoid Limited) in 9 cm Petri plates and left to solidify. The plate's centers were inoculated with 0.5 mm discs of the one-day old culture of *R.solani* and *F.solani* seperately. The treatments considered in quadruplicate. Plates incubated at 28˚C until the control treatment (0%) of each fungus filled the plate. The inhibition percentages were calculated according to Abbott formula (1) *M.javanica* eggs were suspended in water

and equilibrated to 20-25 eggs ml^{-1} then mixed with 3ml of the mentioned formulation dilutions in 5 cm Petri plates in quadruplicate. The plates were incubated for 7 days at 28˚C. The mortality percentage was calculated according to Schneider and Orelli formula **(2)**. One mil of water suspension contained 20-25 second stage larvae of *M.javanica* mixed with 3 ml of each dilution of *P.fluorescens* formulation in 5 cm Petri plates in quadruplicate. The plates were incubated at 28˚C for 7 days. The mortality percentage was calculated according to Schneider and Orelli formula **(1)**. The bioactivity tests were replicated monthly for six months to examine the shelf life of prepared biopesticide formulation. Experimental design: Statistical analysis was performed using Genstat (VSNi) statistical package V. 14 ® software. All the data were tested for normality using Shapiro-wilk test. All experiments data were considered in quadruplicate, The ANOVA statistical analysis with Completely Randomized Design (CRD) was performed and Less Significant Difference (LSD) test used to compare differences among treatment means under statistical significance level of $P \leq 0.05$.

RESULTS AND DISCUSSION

Microorganisms: The morphological identification of isolated fungal pathogens *F.solani* and *R.solani* were confirmed according to Parameter and Whitney (17) and Booth (3). The Nematodes *Meloidogyne javanica* morphological identification was confirmed according to Luc *et al.* (13). *Pseudomonas fluorescens* powder formulation: Significant differences were observed (Fig. 1) in bacterial count numbers among examined powder formulations which represented by superiority of starch and calcium carbonate (35.3, 32.3 × 10^7 CFU ml^{-1}) respectively compare to grind rice and hornwort (18, 16.7 × 10^7 CFU ml^{-1}) respectively with no significant differences between formulations in each group.

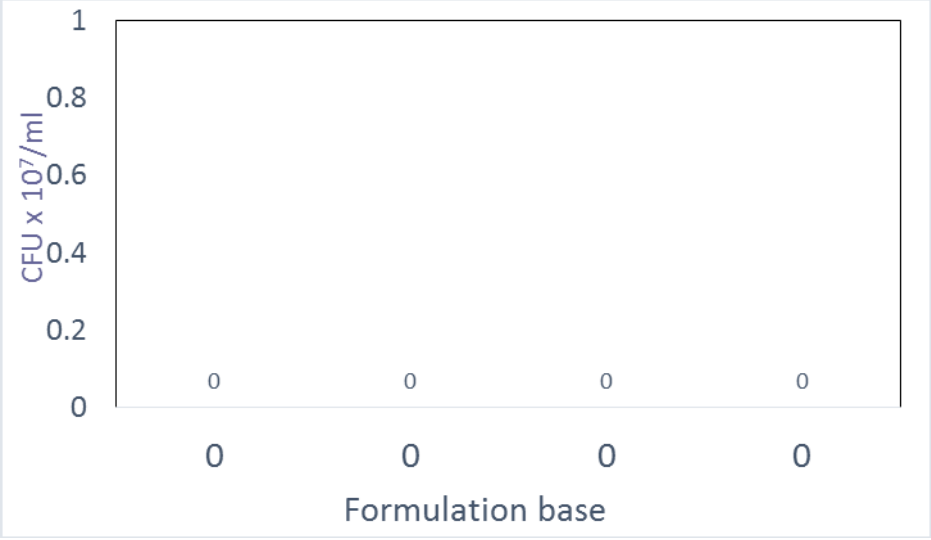

Fig. 1. Comparison among carriers used to formulate biopestisid.

LSD 0.01=5.99

Pseudomonas fluorescens viability in powder formulations: The viability of *P.fluorescens* in $CaCo_3$ powder formulation results (Fig.2a) showed encouraging shelf life period in which the bacterial viability was significantly decreased to the half after one month of storage (4.66×10^9 CFU ml^{-1}) and continued to decreased slightly with no significant differences for the next four months (4.66, 5, 4.33, 3.66 ×10^9 CFU ml^{-1}) respectively, while the lowest viability rate was observed at 180 days of storage (2.33 ×10^9 CFU ml^{-1}) which differed significantly than (30, 60 and 90 days) of storage with no significant differences than (120 and 150 days) of storage respectively. The bacterium decline pattern on starch powder formulation (Fig. 2b) revealed slight reduction in count number lasted for four months (10, 8, 6.66 and 4.33 ×10^9 CFU ml^{-1}) respectively, with significant differences among treatments under P<=0.01 then tended to be stable for the next month (4.33 ×10^9 CFU ml^{-1}) with no significant difference than the fourth month, after that a sharp decline appeared at the end of storage period.

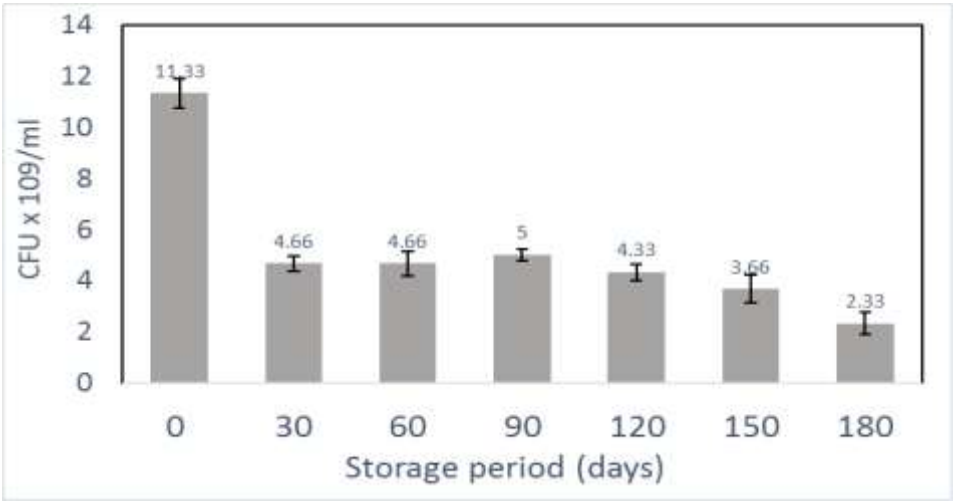

Fig. 2a. The effect of storage period on viability of $CaCo_3$ powder formulation of *P.fluorescens* . LSD 0.01=1.45

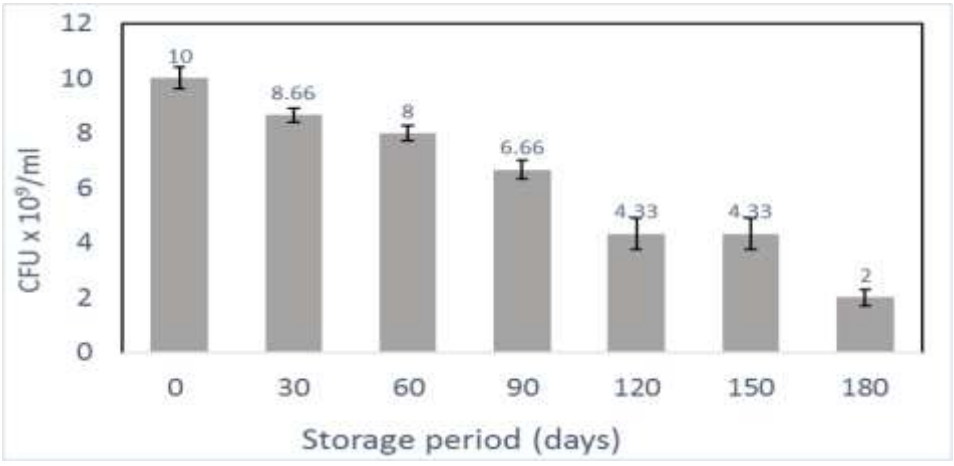

Fig. 2b. The effect of storage period on viability of starch powder formulation of P.fluorescens . LSD 0.01=0.624

Pseudomonas fluorescens bioactivity against fungi: Bioactivity results of calcium carbonate powder formulation exhibited high inhibition to the radial growth rate of *F.solani* (88.88%) *in vitro* during the first storage period which is significantly exceeded the other treatments 30 days treatment 81.47% that in turn surpass 150 and 180 days treatments (79.22, 55.55%) respectively with significant differences between them (Fig. 3).

Fig. 3. Bioactivity of calcium carbonate formulation of *P.fluorescens* on radial growth rate of *F. solani* during different storage periods.
LSD 0.01= 1.86

Calcium carbonate formulation (Fig. 4) presented effective bioactivity *in vitro* against *R.solani*. The results displayed a significant inhibition to the fungal growth at starting date of the experiment (77.78%) excelled all other treatments followed by (30 and 60 days) treatments (74.07 and 73.33%) respectively, which were transcended other followed treatments with no significant differences between them. After 120 days of storage, the formulation activity decreased to 70.37 that significantly exceeded (150 and 180 days) treatments (66.66 and 44.44%) with significant differences between them. *Pseudomonas fluorescens* bioactivity against nematodes: The application of $CaCo_3$ *P.fluorescens* formulation in various concentrations influenced eggs hatching percentage of *M.javanica* significantly comparing with control treatment (Fig. 5). The lowest eggs hatching percentage was at 5 gm l^{-1} treatment (1.33%) that differed significantly than all other treatments except 4 gm l^{-1} treatment (5.33%) which showed no significant difference in eggs hatching percentage.

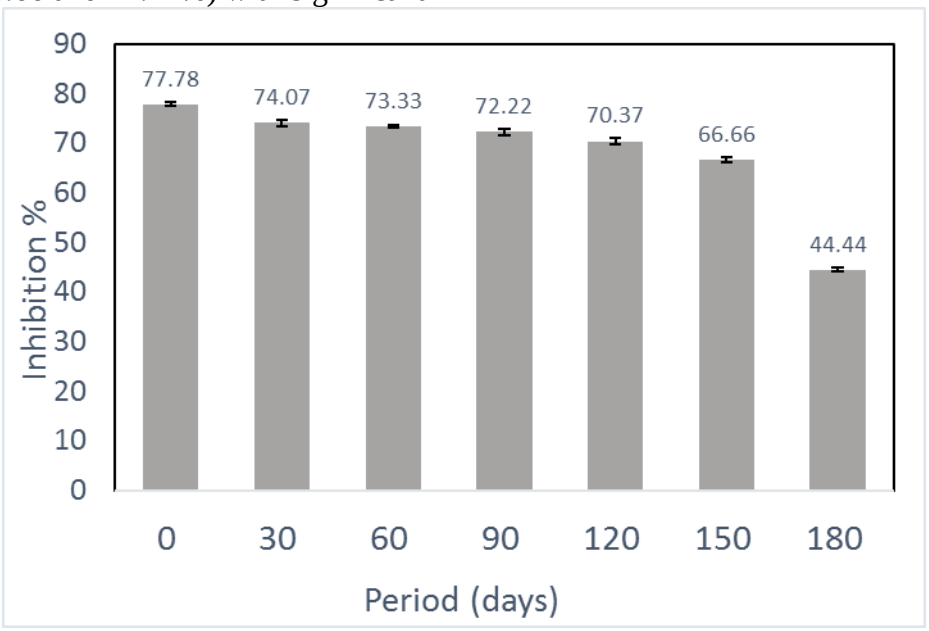

Fig. 4. Bioactivity of calcium carbonate formulation on radial growth rate of *R.solani* during different storage periods. LSD 0.01= 2.51

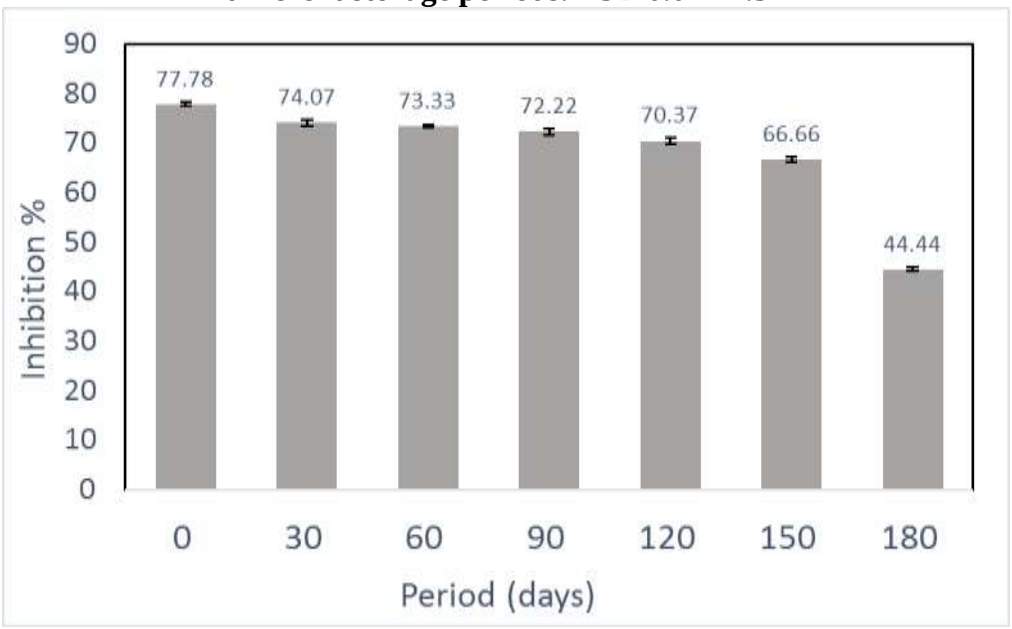

Fig. 5. The effect of various concentrations of *P.fluorescens* $CaCo_3$ formulation on eggs hatching of *M.javanica*. LSD 0.01= 5.53

The results in (Fig. 6) revealed a good ability to kill the second stage larvae (juvenile) of *M.javanica* which presented a superiority of 5 gm l^{-1} treatment (44%) followed by 4 gm l^{-1}

(36.67 %) which were differed significantly than all other treatments although between them while the other treatments 1, 2, 3 gm l^{-1} showed (28, 29.33 and 34.67%) of killing respectively with significant differences between 1 and 3 gm l^{-1} treatments.

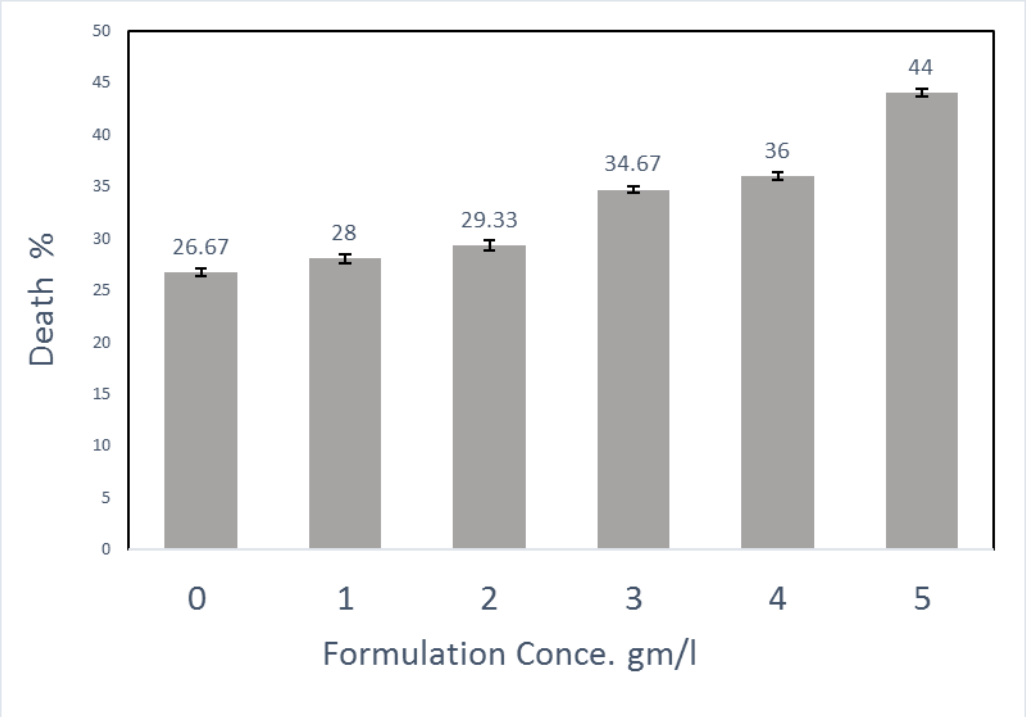

Fig. 6. The effect of various concentrations of *P.fluorescens* $CaCo_3$ formulation on 2nd stage larvae of *M.javanica*. LSD 0.01= 5.37

The carrier base plays an important role in *Pseudomonas* formulation to make it applicable under field conditions, elongate the shelf life, and commercially appropriate (14). The superiority of Starch and $CaCo_3$ in our study may be due to the physical and chemical properties of these compounds to immobilizing and/or encapsulating the bacterium which in turn provides a suitable micro-climate for bacterial individuals to still viable and protected in addition to strengthens the shelf life of formulated bacterium (3, 4, 8, 10, 12, 29). The differences between two examined formulations could be related to their carriers as bacterium viability mostly affected by physical and chemical properties of the carrier such as pH, the capacity of sorption, particle size, surface area, cation exchange, moisture content and other properties (26). The two carriers presented similarity in decline pattern at the last three months of storage which it could return to the functional similarity of both carriers at this storage stage with consideration of chemical and physical differences between them in addition to the bioactivity and/or immobility status of the bacterium itself under microclimate provided by the carrier (4, 15, 26). The bioactivity of the $CaCo_3$ formulation of *P.fluorescens* against *F.solani* and *R.solani* kept high through the experiments' duration in spite of differences among treatments which is proposed to think that the bacterium remained viable throughout that period and its status was good enough to initiate effective antagonistic relationship with the pathogenic fungi which is in turn supporting the viability results of the current study. The mechanism of bioactivity could be related to the secondary antifungal compounds produced by *P.fluorescens* like 2,4-diacetylphloroglucinol (PHL), phenazine (PHE) and siderophore pyoverdin (PYO) which is less effective than the first two compounds in addition to cyclic lipopeptides like tensin, amphisin and hydrogen cyanide (6, 19, 21). The effect of $CaCo_3$ *P.fluorescens* formulation on both eggs hatching percentage and second stage larvae (juvenile) of *M.javanica* tend to be concentration related as it is positively affecting both growth stages with the increase of concentration which it could be related to the ability of *P.fluorescens* to produce HCN, 2,4-Diacetylphloroglucinol (PHL or 2,4 DAPG) which are well-known as a nematocidal compounds at high concentrations, in addition to the probability of

producing extracellular proteases which are functioning as nematocides (16). A similar result previously mentioned by Hamid *et al.* (11) revealed that *P.fluorescens* caused high juvenile mortality according to its ability to produce antimicrobial polyketides like PHL and pyoluteorin PLT. Furthermore, as a biocontrol agent, *P.fluorescens* owned several mechanisms to affect plant pathogens like competition on Fe^{3+}, aggressive colonization of the roots, production of secondary metabolites and plant resistance induction (24)

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