BioFlux ProEnvironmen Original Article
ProEnvironment 15/50 (2022) 287 - 292

Testing *Beauveria bassiana* (Bals.-Criv.) Vuill. (1912) Capacity of Colonizing Pumpkin Plants as an Endophyte, Using a Variety of Inoculation Methods

FEYROZ Ramadan Hassan[1], Samir Khalaf ABDULLAH[2]*, Lazgeen Haji ASSAF[3]

[1] *Plant Protection Department, College of Agricultural Engineering Sciences, Duhok University, Kurdistan region-Iraq*
[2] *Medical Laboratory Technology Department, Alnoor University College, Nineva-Iraq*
[3] *Ministry of Agriculture and Water Resources, Kurdistan region- Iraq*
* Corresponding authore-mail: samir.abdullah@alnoor.edu.iq

Received 10 May 2022; received and revised form 30 May 2022; accepted 12 June 2022; Available online 30 June 2022

Abstract

Beauveria bassiana (Bals.-Criv.) Vuill. (1912) (EE Genbank No. MH374537) isolated from cucumber leaves as natural endophyte and *B. bassiana* (Bals.-Criv.) Vuill. (1912) (ES, Genbank No. MH374538), isolated from soil samples, were transplanted to pumpkin plants via foliar spray, soil drench, and seed treatments, among other ways. The results demonstrated that all three inoculation procedures were efficient in introducing *B. bassiana* (Bals.-Criv.) Vuill. (1912) isolates as endophytes into the pumpkin plant, but to varying degrees. *B. bassiana* (Bals.-Criv.) Vuill. (1912) was recovered from leaves and stems using foliar spray methods and from leaves, stems, and roots by seed treatment inoculation method, and only the stem was separated in drench inoculation method.

Keywords: fungal endophyte, entomopathogenic fungi, cucurbits, biocontrol.

1. Introduction

Endophytic fungicolonize interior plant tissues without causing any symptoms in the host, living inside the intercellular spaces and penetrating living cells, and becoming parasitic only when their hosts are stressed [24]. Some endophytes have been demonstrated to improve their host's growth, abiotic stress tolerance, and pest and disease resistance [14, 16]. There are estimated one million of such distinct endophytic fungal species, the majority of which are ascomycetes and anamorphic fungi [3, 11].

Beauveria bassiana (Bals.-Criv.) Vuill. (1912) has been found as an endophyte in maize [4, 27], potato [2], tomato [17], Western white pineseeds and needles [6], and opium poppy [21]. Colonization by fungal endophytes can be systemic [21,8], localized in plant parts [29, 31], or partitioned [5, 32]. Different taxa of entomopathogenic fungi, on the other hand, have been found to be able to inoculate with plants and become endophytic. Posada et al. (2007) [20] used fungal spore suspensions delivered as foliar sprays, stem injections, or soil drenches to establish the entomopathogenic fungus *B. bassiana* (Bals.-Criv.) Vuill. (1912) in coffee seedlings. Akello et al. (2009) [1] used root and rhizome immersions to introduce *B. bassiana* (Bals.-Criv.) Vuill. (1912) into tissue culture banana plants. To establish *B. bassiana* (Bals.-Criv.) Vuill. (1912) endophytically in the common bean *Phaseolus vulgaris*, Parsa et al. (2013) [22] used two inoculation methods: foliar spray or soil drench with 10 conidia/mL. Greenfield et al. (2016) [7] used soil drench as an inoculation method to transfer *B. bassiana* (Bals.-Criv.) Vuill. (1912) and *Metarhiziumanisopliae* to cassava roots, suggesting that *Metarhiziumanisopliae* has better

capacities to remain in the soil as an endophyte in cassava roots over time. Under laboratory the entomopathogenic fungus *B. bassiana* (Bals.-Criv.) Vuill. (1912) in pumpkin plants using different inoculation procedures.

2. Material and Method

2.1. Preparation of the entomopathogenic fungus*B. bassiana* (Bals.-Criv.) Vuill. (1912) ES was isolated from soil samples collected beneath fallen litter under plants that act as suitable hibernation sites for the sunn pest (the most important insect that attacks wheat crop) from Gara Mountain (N 37 1.51'' E 43 23 34'', 2066 m above sea level) and *B. bassiana* (Bals.-Criv.) Vuill. (1912) EE was isolated from cucumber leaves collected from Amadia district (N 37.0917°, E 43.4877°, 1122 m above sea level) in Duhok city, Kurdistan Region, Iraq.

A commercial animal and fungal DNA preparation kit methodology was used to extract DNA from isolates (Jena Bioscience, Germany). Using universal primers ITS5 and ITS4, genomic DNA was employed as a template for PCR amplification of the ITS region [30]. The sequencing was performed at Macrogen Company, Korea, and submitted to GenBank (GenBank accession MH374537 and MH374538, respectively). Dried and living cultures were deposited at themycology bank, Department of Plant Protection, College of Agricultural Engineering Sciences, Duhok University, BEG22 and BEG23, respectively.

2.2. Testing the potential of the *B. bassiana* (Bals.-Criv.) Vuill. (1912) isolates as an endophyte*Foliar spray and soil drench.* The pumpkin seeds were rinsed with tap water before being surface sterilized by immersing them in 70% ethanol for two minutes, then in 2% sodium hypochlorite for two minutes, followed by 30 seconds in 70% ethanol and rinsing twice in sterile distilled water for two minutes [3]. The seeds were then planted in groups of three in sterilized Peat moss pots in a growth chamber at 25 ± 2°C, 50% RH, and a 12-hour photoperiod.

One week after germination, the two least vigorous seedlings were eliminated [28]. Every 2-3 days, the seedlings were irrigated with sterilized distilled water. The plants were inoculated with suspension of $1x10^7$ conidia/mL of *B. bassiana* (Bals.-Criv.) Vuill. (1912) isolates using two different methods: foliar spray and soil drench, two weeks after planting.

conditions, this work attempted to demonstrate the endophytic establishment of two isolates of

When foliar spray method was applied, a hand atomizer was used to spray the conidial suspension (treatment) or distilled water (control) over the upper surface of leaves. To prevent conidial discharge into the soil, the tops of the pots were covered with aluminum foil before spraying. The plants were covered with plastic bags for 24 hours after spraying to maintain a high level of humidity, which aided fungal infestation.

The soil drench method involved applying 15 mL of conidial suspension (treatment) or distilled water (control) to the surface of the soil at the plant's base using a graduated cylinder. The plants were gently removed and rinsed thoroughly in running tap water after two weeks of treatment.

Five leaflets, five pieces of root, and five pieces of stem were chosen at random from each plant/replicate/ treatment. The samples' surfaces were then sanitized according to Arnold's instructions [3]. After that, each sample (leaflet, stem, and root) was sliced into 3-4 slices and cultured on PDA for two weeks to determine whether *B. bassiana (Bals.-Criv.) Vuill. (1912)* isolates had established themselves as an endophyte within the pumpkin leaf, stem, and root [22].

Pumpkin seeds treatment. According to Arnold, pumpkin seeds were thoroughly cleaned and then surface sterilized [3]. The seeds were immersed in $1x10^7$ spore/mL *B. bassiana* (Bals.-Criv.) Vuill. (1912) isolate conidia suspensions or distilled water (control) for 2 hours, then removed and allowed to dry on filter paper at room temperature.

Pots with sterilized peat moss were put in the growth chamber.

Each pot included three treated seeds, which were planted and allowed to grow. For each fungus, six pots were utilized. After 4 weeks of sowing, the seedling was harvested. Plants were gently taken from the soil and loose soil was shaken off [15].

The seedlings were then surface sterilized after being washed with tap water to remove any remaining peat moss pellets [3]. To evaluate the establishment of *B. bassiana* (Bals.-Criv.) Vuill. (1912) isolates as an endophyte within pumpkin plants by seed treatment, the seedling was cut to acquire sections of leaf, stem, and root, then cultured on PDA medium and incubated for two weeks.

3. Results and Discussions

The quantity and percentage of plants and plant sections positive for the presence of *B.bassiana* (Bals.-Criv.) Vuill. (1912) were calculated from the endophytic potential of both isolates. Fungal inoculation procedures such as foliar spray, soil drench, and seed treatments were all efficient in introducing *B. bassiana* (Bals.-Criv.) Vuill. (1912) into the plant, though at various levels of efficacy.

In comparison to *B. bassiana* (Bals.-Criv.) Vuill. (1912) EE (Genbankacssesion no. MH374537) isolate, *B. bassiana* (Bals.-Criv.) Vuill. (1912) ES (Genbankacssesion no. MH374538) isolate had the maximum colonization in all inoculation procedures.

According to the data presented in Table 1, *B. bassiana* (Bals.-Criv.) Vuill. (1912) ES was recovered from leaves and stems at 26.7% and 6.67% respectively, when foliar spray was applied, but only 13.33% of *B. bassiana* (Bals.-Criv.) Vuill. (1912) EE was recovered from plant leaves.

Table 1. The incidence of *B. bassiana* (Bals.-Criv.) Vuill. (1912) isolates in pumpkin plants using foliar spray, as inoculation method*

Inoculation method/ Isolate	Number of positive plants		Leaves No. ps		Stems No. ps		Roots No. ps	
	+	%	+	%	+	%	+	%
B. bassiana (Bals.-Criv.) Vuill. (1912) EE	1	33.3	2	13.33	0	0	0	0
B. bassiana (Bals.-Criv.) Vuill. (1912) ES	3	100	4	26.7	1	6.67	0	0
Total / Average	4	66.7	6	1.33	1	0.22	0	0

*There were three replicates (plants)/isolate, within each plant (leaves, stems and roots) 15 segments/part were assessed No. ps= number of positive segments/plant part.

Both *B. bassiana* (Bals.-Criv.) Vuill. (1912) isolates did not colonize plant roots. In comparison to *B. bassiana* (Bals.-Criv.) Vuill. (1912) EE isolate, *B. bassiana* (Bals.-Criv.) Vuill. (1912) ES isolate is more efficient for colonization in pumpkin plants, as after four weeks of inoculation, 100% of sprayed plants were colonized by *B. bassiana* (Bals.-Criv.) Vuill. (1912) ES compared to 33.3% for *B. bassiana* (Bals.-Criv.) Vuill. (1912) EE.

According to the data presented in Table 2, *B. bassiana* (Bals.-Criv.) Vuill. (1912) ES and EE were exclusively recovered from stems in soil drench in shares of 26.7% and 6.67%, respectively. Plant leaves and roots were not colonized by any *B. bassiana* (Bals.-Criv.) Vuill. (1912) isolate. The colonization of *B. bassiana* (Bals.-Criv.) Vuill. (1912) ES isolate in pumpkin plants is duplicate (66.6%) to that of *B. bassiana* (Bals.-Criv.) Vuill. (1912) EE isolate (33.3 %).

Table 2. The incidence of *B. bassiana (Bals.-Criv.) Vuill. (1912)* isolates in pumpkin plants using soil drench as inoculation methods*.

Inoculation method/ Isolate	Number of positive plants		Leaves No. ps		Stems No. ps		Roots No. ps	
	+	%	+	%	+	%	+	%
B. bassiana (Bals.-Criv.) Vuill. (1912) EE	1	33.3	0	0	1	6.67	0	0
B. bassiana (Bals.-Criv.) Vuill. (1912) ES	2	66.6	0	0	4	26.7	0	0
Total / Average	3	50.0	0	0	5	0.89	0	0

*There were three replicates (plants)/isolate, within each plant (leaves, stems and roots) 15 segments/part were assessed No. ps= number of positive segments/plant part.

According to the data presented in Table 3, *B. bassiana* (Bals.-Criv.) Vuill. (1912) ES was recovered from stems and roots at 13.3% and 6.67%, respectively, in seed treatment, but only 13.33% of *B. bassiana* (Bals.-Criv.) Vuill. (1912) EE was recovered from plant leaves. *B. bassiana* (Bals.-Criv.) Vuill. (1912) ES isolate colonization in pumpkin plants is equal (33.3%) with *B. bassiana* (Bals.-Criv.) Vuill. (1912) EE isolate colonization in pumpkin plants using this technique of introduction (33.3 %).

In total, 7 out of 90 pieces of sprayed plants with both *B. bassiana* (Bals.-Criv.) Vuill. (1912) isolates yielded positive detection of

1.33% and 0.22% for leaves and stems, respectively, during four weeks of inoculation, stems during the same period of inoculation, and 5 out of ninety 90 segments of plants grown from seed treated with *B. bassiana* (Bals.-Criv.) Vuill. (1912) yielded positive. compared to 4 out of 90 segments of drenched plants yielding positive detection in 0.89% of

Table 3. The incidence of *B. bassiana* (Bals.-Criv.) Vuill. (1912) isolates in pumpkin plants using seed treatment as inoculation methods*.

Inoculation method/ Isolate	Number of positive plants		Leaves No. ps		Stems No. ps		Roots No. ps	
	+	%	+	%	+	%	+	%
B. bassiana (Bals.-Criv.) Vuill. (1912) EE	1	33.3	2	13.3	0	0	0	0
B. bassiana (Bals.-Criv.) Vuill. (1912) ES	1	33.3	0	0	2	13.3	1	6.67
Total / Average	2	33.3	2	0.44	2	0.44	1	0.22

*There were three replicates (plants)/ isolate, within each plant (leaves, stems and roots) 15 segments/part were assessed No. ps= number of positive segments/plant part.

Even though there were no evident patterns between isolates in their ability to remain as endophytes, the results showed that both isolates of *B. bassiana* (Bals.-Criv.) Vuill. (1912) employed in this study have varied capabilities for infection and colonization of pumpkin plant tissues. *B. bassiana* (Bals.-Criv.) Vuill. (1912) has been introduced as an endophyte in a variety of plants utilizing various inoculation methods, such as foliar spraying in potatoes [13], and coating seeds with *B. bassiana* (Bals.-Criv.) Vuill. (1912) conidia in tomatoes [18, 26].

To establish *B. bassiana* (Bals.-Criv.) Vuill. (1912) endophytically in the common bean, Parsa et al. (2013) [19] used two inoculation methods: foliar spray or soil drench with 10 conidia/mL (*Phaseolus vulgaris*). As inoculation methods, *B. bassiana* (Bals.-Criv.) Vuill. (1912) was delivered to cassava roots by soil drench [7]. *B. bassiana* (Bals.-Criv.) Vuill. (1912) has been found to colonize a variety of cucurbitaceous hosts in earlier research. When inoculated as conidia, Gurulingappa et al. (2010) [8] found that *B. bassiana* (Bals.-Criv.) Vuill. (1912) colonized the leaves of six crop plants, including pumpkin.

Jaber and Salem (2014) [12] used foliar spray to introduce *B. bassiana* (Bals.-Criv.) Vuill. (1912) into squash plants to provide protection against the Zucchini Yellow Mosaic Virus (ZYMV). According to Resquin-Romero et al. (2016) [23], spraying melon plants with a conidial suspension of the entomopathogen *B. bassiana* caused temporal endophytic colonization of the leaves, which resulted in increased death of *Spodoptera littoralis* larvae.

After 10 days of artificial seed inoculation with conidial solution of *B. bassiana* (Bals.-Criv.) Vuill. (1912), Shaalan and Ibrahim (2018) [25] demonstrated effective colonization of plant tissues in cucumber seedlings, achieving 85.3% recovery in cotyledon and stem, and 50% recovery in roots. *B. bassiana* (Bals.-Criv.) Vuill. (1912) was found naturally on cucumber leaves in Iraq, according to Hassan et al. (2019a) [9]. Hassan et al. (2019b) [10] tested the pathogenicity of an endophytic *B. bassiana* (Bals.-Criv.) Vuill. (1912) isolated from cucumber leaves against several stages of the squash beetle *Epilachna chrysomelina* (F.) in the field and in the laboratory. Rajab et al. (2020) [22] demonstrated the ability of *B. bassiana* (isolate B195) to colonize, survive, and develop systemically in cucumber plant stem, leaf, petiole, and root 30 days after artificial inoculation under indoor settings.

4. Conclusion

Both isolates of *B. bassiana* (Bals.-Criv.) Vuill. (1912) employed in this study have varied capabilities for infection and colonization of pumpkin plant tissues. The establishment of the entomopathogenic *B. bassiana (*Bals.-Criv.) Vuill. (1912) as an endophyte in pumpkin plant tissues may play a role in pest population regulation and control.

Acknowledgments. This research was supported by the Department of Plant Protection, College of Agricultural Engineering Sciences, University of Duhok, Duhok, Iraq, which is greatly appreciated. We express our sincere gratitude to the Razga company for the financial support which made thisconference possible.

References

[1] Akello J., T. Dubois,D. Coyne,K. Kyamanywa, 2009, The effects of *Beauveria bassiana* dose and exposure duration on colonization and growth of tissue cultured banana (*Musa* sp.) plants. Biol. Control 49,6-10.

[2] Alsaoud N, A., D.A.Nammour,Y.A. Ali, 2017, The endophytic ability of *Beauveria bassiana* (balsam)Vuill. In potato plant *Solanum tuberosum* L. Al Baath Univ.J.39,139-168.

[3] Arnold A.E.2007, Understanding the diversity of foliar endophytivprogress.,challenges, and frontiers. Fungal Biology Reviews 21, 51–66.

[4] Arnold, A. E., L.C.Lewis, 2005, Ecology and evolution of fungal endophytes, and their roles against insects. In: Insect-Fungal Associations: Ecology and Evolution.pp.74-96.Vega, JR, M Blackwell (Eds.). Oxford University Press, New York,USA.

[5] Behie S.W.,S.J. Jones, M.J. Bidochka, 2015, Plant tissue localization of the endophytic insect pathogenic fungi *Metarhizium* and *Beauveria*. Fungal Ecol. 13, 112–119.

[6] Ganley R.J., G. Newcombe, 2005, Fungal endophytes in seeds and needles of *Pinus monticola*. Mycological Research 110, 318–327.

[7] Greenfield M., M.I.Gómez-Jiménez, V. Ortiz,F.E. Vega,M. Kramer, S. Parsa,2016,*Beauveria bassiana* and *Metarhiziumanisopliae* endophytically colonize cassava roots following soil drench inoculation. Biol. Control 95, 40–48

[8] Gurulingappa P., G.A. Sword, G. Murdoch, P.A. McGee. 2010. Colonization of crop plants by fungal entomopathogens and their effects on two insect pests when in planta. Biol. Control 55, 34–41.

[9] Hassan F.R., S.K. Abdullah, L.H. Assaf, 2019a, Molecular identification and biomass production of an endophytic *Beauveria bassiana* isolated from cucumber leaves in Iraq. J. Univ. Duhok (Agri. and Vet. Sciences) 22,38-47.
https://doi.org/10.26682/cajuod.2020.22.25

[10] Hassan F.R.,S.K. Abdullah,L.H. Assaf, 2019b, Pathogenicity of the entomopathogenic fungus, *Beauveria bassiana* (Bals)Vuill. Endophytic and a soil isolate against the squash beetle, *Epilachnachrysomelina* (F.) (Coleoptera: Coccinellidae). Egypt. J. Biol. Pest Control 29,74. https://doi.org/10.1186/s41938-019-0169-x

[11] Huang Y., J.Wang, G.LI, Z.Zheng, W.Su, 2001, Antitumor and antifungal activities in endophytic fungi isolated from pharmaceutical plants *Taxus mairei, Cephalataxusfortunei* and *Torreya grandis*. FEMS Immunol. Med. Mic. 31: 163–167.

[12] Jaber L.R., N.M.Salem, 2014, Endophytic colonization of squash by the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales) for managing Zucchini yellow mosaic virus in cucurbits. Biocontrol Sci. Technol. 24,1096-1109.

[13] Jones K.D, 1994, Aspects of the biology and biological control of the European corn borer in North Carolina. Ph.D. thesis, Department of Entomology, North Carolina State University.

[14] Kabaluke J.T., J.D. Ericsson, 2007, *Metarhiziumanisopliae* seed treatment increases yield of field corn when applied for wireworm control, Agron. J., 99,1377-1381.

[15] Keyser C.A., K.Thorup-Kristensen,N.V. Meyling, 2014,*Metarhizium*seed treatment mediates fungal dispersal via roots and induces infections in insects.Fungal Ecol. 11, 122-131.

[16] Kuzhuppillymyal-Prabhakarankutty L., P. Tamez-Guerra, R. Gomez-Flores, M.C. Rodriguez-Padilla, , M.J, EK- Ramos, 2020, Endophytic *Beauveria bassiana* promotes drought tolerance and early flowering in corn. World J. Microbiol. Biotechnol., 36,47, https://doi.org/10.1007/s112747-020-02823-4.

[17] Omukoko C., L. Turoop, 2017, Colonization of tomato varieties by *Beauveria bassiana* isolates in the screen house. Int.J.Sci.Res. 6:1024-1028. https://doi.org/10.21275/ART20175332

[18] Ownley B. H., M.R. Grifn,W.E. Klingeman,K.D. Gwinn,J.K. Moulton,R.M. Pereira, 2008,*Beauveria bassiana*: endophytic colonization and plant disease control. J InvertebrPathol. 98, 267–70.

[19] Parsa S., V.Ortiz,F.E. Vega,F.E, 2013, Establishing fungal entomopathogens as endophytes: towards endophytic biological control. J. Vis. Exp. 74, 50360.

[20] Posada, F., M.C.Aime, S.W.Peterson,S.A.Rehner, F.E. Vega, 2007, Inoculation of coffee plants with the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales). Mycol. Res 111,748–757.

[21] Quesada-Moraga E., B.B.Landa,J. Mun˜oz-Ledesma,R.M. Jime´nez-Dı´az, C.Santiago,A. lvarez,2006, Endophytic colonization of *Opium poppy, Papaver somniferum*, by an entomopathogenic *Beauveria bassiana* strain. Mycopathologia 161, 323–329.

[22] Rajab L.,M. Ahmed, I. Gazal, 2020, Endophytic establishment of the fungal entomopathogen, *Beauveria bassiana* (Bals.)Vuil., in cucumber plants. Egypt. J. Biol. Pest Control 30,143,

https://doi.org/10.1186/s41938-020-00344-8.

[23] Resquin-Romero G., I.Garrido-Jurado,C. Delso, A. Rios-Moreno, E. Quesada-Moraga, 2016, Transient endophytic colonization of plants improves the outcome of foliar applications of mycoinsecticides against chewing insects, J. Invertebr. Pathol. 136, 23-31.

[24] Schouten A., 2019, Endophytic fungi: definition, diversity, distribution and their significance in plant life. In: Endophytic biotechnology: Potential for agriculture and pharmacology Schouten A. (Eds.), CABI Biotechnology series, https://doi.org/10.1079/9781786399427.0006

[25] Shaalan R., L. Ibrahim, 2018, Entomopathogenic fungal endophytes: can they colonize cucumber plants? In: Proceedings of the IX International Agricultural Symposium "Agrosym 2018" pp 853-860, University of East Sarajevo, Jahorina, Bosnia and Herzegovina.

[26] Silva A.C.L., G.A. Silva,P.H.N. Abib,A.J. Carolino,R.I. Samuels, 2020, Endophytic colonization of tomato plants by the entomopathogenic fungus *Beauveria bassiana* for controlling the South American tomato pinworm, *Tutaabsoluta*. CABI Agriculture and Bioscience. 1:3 https://doi.org/10.11861/s43170-020-00002-x.

[27] Vakili NG., 1990, Biocontrol of stalk rot in corn, In: Proceedings of the Forty-fourth Annual Corn and Sorghum Industry Research Conference. Pp.87-105 December 6–7, 1989, Chicago, IL. American Seed Trade Association, Washington, DC.

[28] Vega F.E., N.V. Meyling, J.J.Luangsa-ard,M. Blackwell, 2012, Fungal entomopathogens. In: Insect Pathology, second ed. pp. 171–220.Vega FE,HK Kaya (Eds.), Academic Press, San Diego.

[29] Wearn J.A., B.C.Sutton,N.J. Morley,A.C. Gange, 2012, Species and organ specificity of fungal endophytes in herbaceous grassland plants. J. Ecol. 100: 1085–1092.

[30] White T., T. Bruns,S. Lee,J. Taylor,1990, Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics.In: PCR protocols: a guide to methods and applications. pp

[31] Yan J.F., S.J. Broughton,S.L. Yang, A.C. Gange, 2015, Do endophytic fungi grow through their hosts systemically? Fungal Ecol. 13, 53–59.

[32] Zambell C.B., J.F.White, 2014, In the forest vine *Smilax rotundifolia*, fungal epiphytes show site-wide spatial correlation, while endophytes show evidence of niche partitioning. Fungal Diversity 75, 279–297.