The Vegetative Growth Response of Detam Soybean Varieties towards *Bacillus subtilis* and *Trichoderma* sp. Applications as Bio-fertilizer

Agus Miftakhurrohmat[1] and *Sutarman* [1,*]

[1] Department of Agrotechnology, Faculty of Science and Technology, Universitas Muhammadiyah Sidoarjo

Abstract. This study aims to determine the effect of bacterial isolates of *Bacillus subtilis* Bs-Sdj-01 and *Trichoderma* sp. Tc-Jro-02 isolates as biological fertilizer in plant growth until the end of the vegetative phase. The experiment was arranged factorially in a Completely Randomized Design (CRD) with each factor being the application of *B. subtilis* consisting of and without *B. subtilis* and the Trichoderma application consisting of with and without Trichoderma; the experiment was repeated four times. The observed variables were: plant height, stem diameter, number of leaves, leaf area, stover dry weight, root dry weight, and *B. subtilis* population at the end of the observation. Data were analyzed using with ANOVA and HSD tests at the 5% level. The combination of *Trichoderma* sp. and *B. subtilis* bacteria produce the highest increase in plant height, stem diameter, number of leaves, leaf area, stover dry weight, and root dry weight of soybean plants up to 35 days after planting and increase B. *subtilis* population grew from 10^{10} CFU.g^{-1} to be 4.43x10^{11} CFU.g^{-1}. The presence of *Trichoderma* supports the activity of *B. subtilis* in the rhizosphere of the Detam variety soybean plantation.

1 Introduction

Currently, various ways have been taken in order to realize Indonesia's independence in meeting the various national needs of soybeans, not only for tofu and *tempe* production but also for various other industrial needs. Black soybean is one of the soybean plant variants whose seeds are used as raw material in the soy sauce industry.

On the other hand, to increase soybean production, extensification land for soybean planting is carried out by utilizing dry land. However, challenges are always found, including low soil organic matter, soil acidity, and attack by plant pests. Thus the dry land soils have low fertility both chemically, physically, and biologically.

The ability to produce and plant productivity is very much determined by the success of plants in passing the critical phase, namely the vegetative phase [1] including those grown in conditions between strong environmental pressures on dry land. Dryland environmental stress is represented by low organic matter content, limited nutrient availability, and biologically unfavourable rhizosphere conditions for plant growth [2].

Efforts to overcome environmental stress on plants include utilizing effective microbes that can help plants and improve soil fertility. From the fungi group, Trichoderma spp. whose role is to provide protection for plants from disease in the rhizosphere [3-4]. Extracellular

* Corresponding author: sutarman@umsida.ac.id

compounds that play a role in promoting growth for plants are produced by microorganisms from a group of effective bacteria including Bacillus spp. [5]. Effective microbial support of Trichoderma and Bacillus bacteria in the rhizosphere can increase soil fertility and nutrient availability as well as promote vegetative growth and plant health protection [6]. However, there is not much information on the use of these two types of microbes isolated from dry land where soybean plants are commonly grown or on dry land that has never been cultivated with soybean cultivation, especially black soybean.

On marginal dry land, especially in plantation and plantation areas where land is available between the rows of perennials, land conditions that often receive sunlight with low light intensity are often found in addition to the lack of various important elements for plants. Certain soybean varieties cultivated inland with low light intensity and low soil fertility are able to grow optimally with the help of effective microbes in the soil. Fungi *Trichoderma* sp. and *B. subtilis* bacteria have been shown to be effective in helping Dena soybean varieties in dealing with low light intensity stresses up to 60% shade intensity above that recommended by previous researchers [7]. Performance of *Trichoderma* even able to show a good morphological response, especially by maintaining the intensity of the stomata [8], thus ensuring optimal vegetative growth. On the other hand, testing of effective microbial applications, especially *Trichoderma* and *Bacilus*, to support the growth of soybean from other types, which are grown on marginal dry land even though it has sufficient light, needs to be done.

Given *Trichoderma* spp. and *Bacillus* spp. having the same niche in the rhizosphere [9], it is necessary to reveal the extent of activity and possible interactions between these two types of effective microbes in making a real contribution to plant growth.

This study aims to determine the effect of the interaction between bacterial isolates of *B. subtilis* and *Trichoderma* sp. on the growth of Detam black soybean plants until the end of the vegetative phase.

2 Methods

2.1 Preparation and planting

The research was carried out in the Microbiology laboratory and Green House of the Universitas Muhammadiyah Sidoarjo (UMSIDA) in March-July 2019.

Trichoderma sp. isolate Tc-Jro-01 and *Bacillus subtilis* isolate Bs-Sdj-01 used in this experiment was a collection of the UMSIDA. *Trichoderma* fungus isolates were propagated in PDA-chloramphenicol media [10] within 14 days of incubation. The culture of the harvested isolates was crushed and mixed with water to produce a suspension with a population density of 10^8 colony-forming units per ml ($CFU.ml^{-1}$). On the other hand, *B. subtilis* isolates were propagated on NA media, after seven days the incubation period the cultures were harvested and grown into sterile corn granules for seven days. As a carrier medium in applications as a bio fertilizer is compost of husks that are ripe and sterile. Both the *Trichoderma* suspension and *B. subtilis* propagules in corn granules are mixed with husk compost and the composition is adjusted so that the husk flour which has been used as a bio fertilizer has a density of 10^8 $CFU.gr^{-1}$ for *Trichoderma* and 10^{10} $CFU.gr^{-1}$ for *B. subtilis*. Each bio fertilizer was applied as initial fertilization in soybean plants.

The soil used as a planting medium is from land with an altitude of ± 8.0 m asl. in Jiken Village, Tulangan District, Sidoarjo Regency, East Java Province with basic characteristics: pH (H2O) 7.05, C-organic 0.56%, C/N ratio 14, CEC 29.64, and dusty clay texture. The soil used is sterilized by an autoclave at 120 ^{0}C 1 atm for 30 minutes. This sterilization is to free all microbes including root nodules that are present in the soil. Thus in this experiment, there was no symbiosis between the roots and the root nodules bacteria. Soil that has been sterile is placed in polybags with a capacity of five kg of soil. Into each polybag was poured bio fertilizer *Trichoderma* and/or *B. subtilis* according to the treatment with a dose of 200 g $polybag^{-1}$ and stirred to mix evenly. Thus the population of *Trichoderma* sp. and *B. subtilis* in

the soil of the planting medium of $0.4x10^7$ CFU.g^{-1} and $0.4x10^9$ CFU.g^{-1}, respectively.

The soybean used in this experiment was black soybean variety Detam 3 Prida which was released based on the Decree of the Minister of Agriculture No. 4385/Kpts/SR.1206/ 2013. Surface sterilization of soybean seeds using a 50% alcohol solution for three seconds and rinsed with sterile water three times. After draining, three seeds are planted into each polybag and one good vigor sprout will be maintained.

2.2 Experimental design

In this study, the experiment was arranged factorial with two factors, namely: (i) *B. subtilis* application consisting of without and with *B. subtilis*, and (ii) application of Trichoderma consisting of without and with Trichoderma. The four treatment combinations obtained were repeated four times, resulting in 16 experimental rules arranged in a completely randomized design (CRD). Each experimental unit consisted of four polybags containing four plants each. The observed variables were: plant height (cm), stem diameter (mm), number of leaves, and leaf area (cm^2) at 14-35 days after planting (DAP), stover dry weight (gr), root dry weight (gr), and the population of *B. subtilis* (CFU.gr^{-1}) at the end of the observation.

2.3 Statistical analysis

Data were analyzed by ANOVA at the 5% level. Each treatment mean was compared with the control (without *B. subtilis* and without *Trichoderma*) to determine the percentage increase or decrease in control [11].

3 Results and Discussion

3.1 Results

3.1.1 The plant height

The results of the analysis of variance showed that the effect of the interaction between *B. subtilis* and Trichoderma was very significant ($p < 0.01$) on soybean plant height at 28 and 35 days after planting (DAP) based on the results of the analysis of variance. Each treatment factor did not show a significant effect ($p > 0.05$) on the plant stem diameter at all observation times. The growth pattern of soybean stem diameter 28-35 DAP in each treatment is shown in Figure 1.

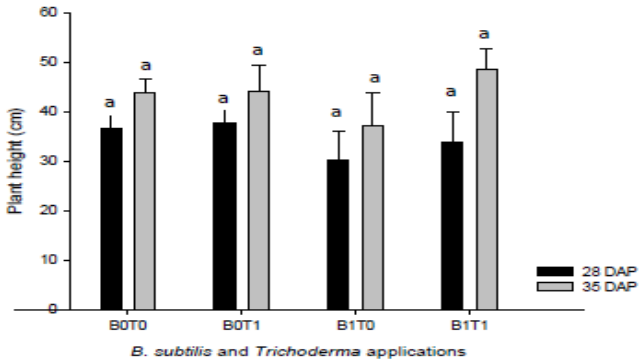

Fig. 1. Growth height of soybean varieties Detam 28-35 DAS DAP (cm). Different lowercase letters above the bars of the same color (same time of observation) showed a significant difference between treatments for the concentration of *B. subtilis* cells according to the HSD test ($p<0.05$).

The average effect of the application of *B. subtilis* and fungi *Trichoderma* sp. The height growth of soybean varieties Detam and the percentage of increase and decrease in control (Without Bacillus - Without Trichoderma) are shown in Table 1.

Table 1. The average effect of *B. subtilis* and *Trichoderma* sp. to the height of Deta soybean varieties and the percentage increase or decrease compared to control at 28-35 DAP

Application treatment	28 DAP		35 DAP	
	Plant height (cm)	Δx (%)	Plant height (cm)	Δx (%)
Without *Bacillus* – Without *Trichoderma* (B0T0) (control)	23.25	-	43.70	-
Without *Bacillus* - *Trichoderma* (B0T1)	27.45	18.06%	43.93	0.51%
Bacillus - Without *Trichoderma* (B1T0)	22.38	-3.76%	37.23	-14.82%
Bacillus - *Trichoderma* (B1T1)	18.38	-20.97%	48.43	10.81%

Δx: Increase or decrease (-) compared to control

3.1.2 Rod diameter

The effect of the interaction between *B. subtilis* and *Trichoderma* was very significant ($p<0.01$) on the stem diameter of soybean plant height from 28 to 35 DAP (Figure 2), but each treatment factor was not significant ($p> 0.05$).

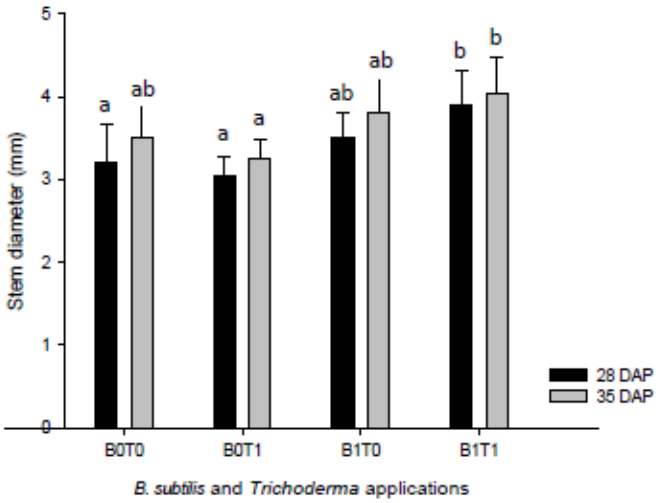

Fig. 2. The average stem diameter of soybean varieties Detam 28-35 DAP (mm). Different lowercase letters above the bars of the same color (same time of observation) showed a significant difference between treatments for the concentration of *B. subtilis* cells according to the HSD test ($p<0.05$)

Table 2. The average effect of *B. subtilis* and *Trichoderma* sp. to the stem diameter of Detam soybean varieties and the percentage increase or decrease compared to control at 28-35 DAP

Application treatment	28 DAP		35 DAP	
	stem diameter (mm)	Δx (%)	stem diameter (mm)	Δx (%)
Without *Bacillus* – Without *Trichoderma* (B0T0) (control)	3.20	-	3.50	-
Without *Bacillus* - *Trichoderma* (B0T1)	3.06	-4.69%	3.26	-6.86%
Bacillus - Without *Trichoderma* (B1T0)	3.50	9.37%	3.80	8.57%
Bacillus - *Trichoderma* (B1T1)	3.90	21.88%	4.06	16.00%

Δx: Increase or decrease (-) compared to control

The average effect of application of *B. subtilis* and fungi *Trichoderma* sp. The growth of stem diameter of soybean varieties Detam and the percentage of increase and decrease in control (B0T0) are shown in Table 2.

3.1.3 Number and area of leaves

B. subtilis and *Trichoderma* each had no significant effect on the number of leaves, but the effect of the interaction was very significant (p <0.01) on 28 and 35 DAP (Figure 3). Meanwhile Table 3 shows the percentage of increase and decrease in the control (B0T0).

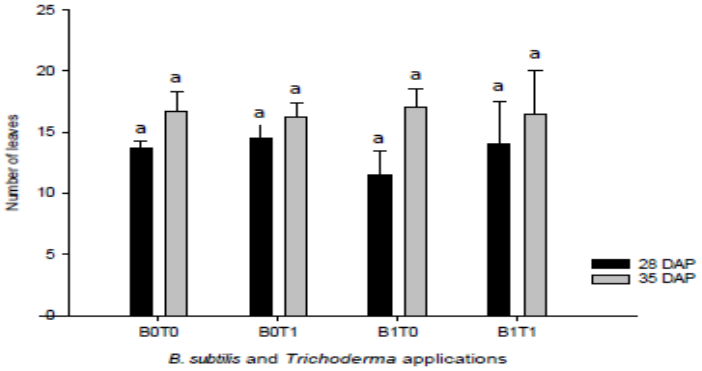

Fig. 3. The average number of leaves of soybean Detam varieties 28-35 DAP. Different lowercase letters above the bars of the same color (same time of observation) showed a significant difference between treatments for the concentration of *B. subtilis* cells according to the HSD test (p<0.05)

Table 3. The average effect of *B. subtilis* and *Trichoderma* sp. on the number of leaves of Detam soybean plants and the percentage increase or decrease compared to control at 28-35 DAP

Application treatment	28 DAP		35 DAP	
	number of leaves	Δx	number of leaves	Δx
Without *Bacillus* – Without *Trichoderma* (B0T0) (control)	13.75	-	16.75	-
Without *Bacillus* - *Trichoderma* (B0T1)	14.50	5.45%	16.25	-2.99%
Bacillus - Without *Trichoderma* (B1T0)	11.50	-16.36%	17.00	1.49%
Bacillus - *Trichoderma* (B1T1)	14.00	1.82%	16.50	-1.49%

Δx: Increase or decrease (-) compared to control

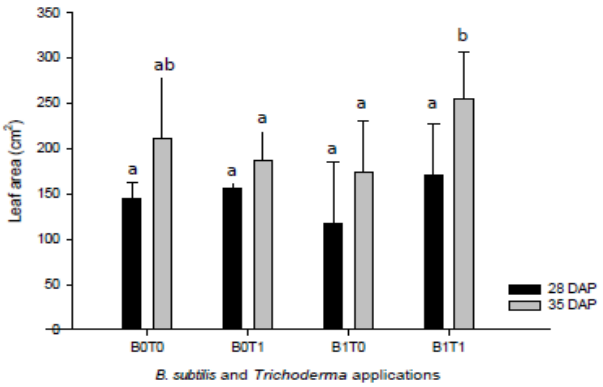

Fig. 4. Growth of leaf area of Soybean varieties Detam 28-35 DAP (cm^2). Different lowercase letters above the bars of the same color (same time of observation) showed a significant difference between treatments for the concentration of *B. subtilis* cells according to the HSD test (p<0.05)

The effect of interactions between *B. subtilis* and *Trichoderma* sp. on leaf area was very significant ($p < 0.01$) at 28 and 35 DAP, but they did not significantly affect leaf area, respectively. Leaf area growth from 28 to 35 DAP is shown in Figure 4.

The average effect of the application of *B. subtilis* and fungi *Trichoderma* sp. The growth of the leaf area of the Detam variety soybean plant and the percentage of increase and decrease in control (B0T0) is shown in Table 4.

Table 4. The average effect of *B. subtilis* and *Trichoderma* sp. on leaf area of Detam soybean varieties and the percentage increase or decrease compared to control at 28-35 DAP

Application treatment	28 DAP		35 DAP	
	Leaf area (cm^2)	Δx (%)	Leaf area (cm^2)	Δx (%)
Without *Bacillus* – Without *Trichoderma* (B0T0) (control)	144.0		211.13	
Without *Bacillus* - *Trichoderma* (B0T1)	155.25	7.44%	186.50	-11.66%
Bacillus - Without *Trichoderma* (B1T0)	116.50	- 9.38%	173.38	-17.88%
Bacillus - *Trichoderma* (B1T1)	170.50	17.99%	254.25	20.43%

Δx: Increase or decrease (-) compared to control

3.1.4 Plant dry weight

Effect of interactions between B. subtilis and Trichoderma sp. to dry weight of stover and root were very significant ($p < 0.01$). Table 5 shows the mean effect of each treatment combination as well as the percentage of increase and decrease in control (B0T0).

Table 5. The average effect of *B. subtilis* and *Trichoderma* sp. to dry weight of stover and root dry weight of soybean plants of Detam variety and the percentage of increase or decrease compared to control at 28-35 DAP

Application treatment	28 DAP		35 DAP	
	Stover dry weight (g)	Δx (%)	Root dry weight (g)	Δx (%)
Without *Bacillus* – Without *Trichoderma* (B0T0) (control)	0.88	-	0.15	-
Without *Bacillus* - *Trichoderma* (B0T1)	1.04	17.56%	0.30	105.17%
Bacillus - Without *Trichoderma* (B1T0)	2.83	220.68%	0.43	194.83%
Bacillus - *Trichoderma* (B1T1)	1.00	13.31%	0.30	103.45%

Δx: Increase or decrease (-) compared to control

3.1.5 B. subtilis population density

The results of the calculation of the average population of *B. subtilis* are presented in Table 6. The density of the population of *B. subtilis* without *Trichoderma* was 4.05×10^{11} CFU g^{1}, while in the growing media conditions given *Trichoderma* sp. obtained a population means of 4.43×10^{11} CFU.g^{-1}.

Table 6. Average effect of *B. subtilis* and *Trichoderma* sp. against the population density of *B. subtilis* at 35 DAP

Application treatment	*B. subtilis* population (CFU g^{-1})
Without a *Bacillus* – Without *Trichoderma* (B0T0)	0
Without *Bacillus* - *Trichoderma* (B0T1)	0
Bacillus - Without *Trichoderma* (B1T0)	4.05×10^{11}
Bacillus - *Trichoderma* (B1T1)	4.43×10^{11}

3.2 Discussion

The vegetative growth of black soybean (Detam variety) in response to the activity of *Trichoderma* and *B. subtlis* in the rhizosfer showed a very large increase of about twofold compared to the treatment without *B. subtilis* and *Trichoderma* sp.

Trichoderma uses mostly organic matter as a substrate for its activities [12]. The decomposition of organic matter by *Trichoderma* with the help of various degrading enzymes [13] that it produces will contribute nutrients to meet the needs of plant growth [14] whose responses are shown mainly in the form of stover dry weight and root dry weight (Table 5).

B. subtilis in this experiment significantly showed evidence of its ability to increase plant growth compared to control (without *B. subtilis* and *Trichoderma*). *B. subtilis* was able to increase the average stem diameter up to 8.57% (Table 2) and plant biomass of 220.68% (dry weight of stover) and 194.83% (dry weight of roots) (Table 5) at the end of the observation (35 DAP). These bacteria produce secondary metabolites which can stimulate plant growth [15].

Trichoderma not only acts as a fertilizer supplier because of its ability to degrade organic matter, releasing nutrients for plant needs and helping plant growth [7, 16] but also secondary metabolites [17-18] and various growth factors [19] which helps plant vegetative growth. Meanwhile, *B. subtilis* can produce several secondary metabolites, among which act as plant growth-regulating compounds [8]; in addition, these bacteria are capable of producing lipopeptides and antibacterial proteins [20]. Thus *B. subtilis* not only provides plant protection from pathogenic disturbances but also promotes the growth of soybean plants [21].

The real interaction between the effect of *Trichoderma* and *B. subtilis* on all vegetative growth variables shows a synergy that results in the growth response of the Detam black soybean plant variety in this experiment. The combination of these two microbes resulted in an increase in plant height (Figure 1) up to 10.81% at the end of the observation in Table 1), stem diameter (Figure 2) up to 16.00% (Table 2), leaf area (Figure 4) up to 24.43% (Table 4), as well as stover dry weight and dry weight. roots 13.31% and 103.45%, respectively (Table 5). In observing the population of *B. subtilis*, it appears that the density of these bacterial cells in rhizosphere soil conditions contains *Trichoderma* sp. It turns out that the population is $4.43x10^{11}$ CFU.g^{-1}, while the population without *Trichoderma* only reaches $4.05x10^{11}$ CFU.g^{-1} (Table 6). This shows that *B. subtilis* is not under the stress of *Trichoderma*. *Trichoderma* activity can promote beneficial microbial activity in the rhizosphere [22] including *B. subtilis* in this experiment. *Trichoderma* does not interfere with the stability of bacterial cells, with the chitinase enzyme that is produced [5] this fungus usually disrupts the stability of the fungal cell walls, especially those with pathogenic properties.

The high percentage increase in stover dry weight and root dry weight was not only a result of the role of *B. subtilis* and *Trichoderma* sp but also as a form of optimal response from plants. Soybean is responsive to light [9] which is manifested in optimal sugar production at the end of the photosynthesis process and plays an important role in the process of young tissue growth [23] and the process of transition from young plants to mature plants in the vegetative phase [10].

4 Conclusion

The interaction effect of *Trichoderma* sp Tc-Jro-02 and *Bacillus subtilis* Bs-Sdj-01 isolates was very significant on plant height, stem diameter, number of leaves, leaf area, stover dry weight, and root dry weight of Detam soybean at 35 days after planting. The combination of these two biofertlation agents increases the population of *B. subtilis* in the rhizosphere from 10^{10} CFU.g^{-1} to $4.05x10^{11}$ CFU.g^{-1}.

Giving *B. subtilis* bacteria alone does not show the role of helping plants optimally. The presence of *Trichoderma* supports the activity of *B. subtilis* in the rhizosphere of Detam soybean plantations; the combination of these two types of effective microbes can be further developed to be formulated into a biofertilizer for plant fertilization action.

Acknowledgement. The author would like to thank the Ministry of Research and Technology of the Republic of Indonesia for providing financial support for this research through the 2020 Higher Education Excellence Basic Research Grant (PDUPT).

References

1. P. Basuchaudhuri. *Indian J. Plant Sci.* **5**, 25–38 (2016)
2. Sutarman, A.E. Prihatiningrum, A. Sukarno, and A. Miftahurrohmat. *IOP Conf. Ser. Mater. Sci. Eng.* **420**, 12064 (2018)
3. A. Singh, N. Shukla, B.C. Kabadwal, A.K. Tewari, and J. Kumar. *Int. J. Curr. Microbiol. App. Sci.* 7(2): 2382–2397 (2018)
4. S. Leylaie and D. Zafari. *Front. Microbiol.* **9**: 1484 (2018)
5. C. Li, J. Yu, L. Gan, J. Sun, C. Wang, Q. Wang, S. Chen, and Y. Yang. *Open J. Appl. Sci.* **8**: 518–531 (2018)
6. C. Buysens, V. César, F. Ferrais, H. Dupré de Boulois, and S. Declerck. *Appl. Soil Ecol.* **105**, 137–143 (2016)
7. A. Miftahurrohmat and Sutarman. *IOP Conf. Ser. Mater. Sci. Eng.* **420**, 12069 (2018)
8. A. Miftahurrohmat, F.D. Dewi, and Sutarman. *IOP J. Phys. Conf. Ser.* **1232**, 12043 (2019)
9. S. Youssef, K. Tartoura, and G.A. Abdelraouf. *Biological Control* **100**, 79–86 (2016)
10. A. Wachid and Sutarman. *IOP Conf. Ser.* **1232** 012020 (2019)
11. A Miftahurrohmat and Sutarman. *IOP Conf. Ser. Mater. Sci. Eng.* **821** 012002 (2020)
12. X. Hu, D.P. Roberts , L. Xie, J.E. Maul, C. Yu, Y. Li, Y. Zhang, L. Qin, and X. Liao. *Phytopathology* **105**, 1325–1333 (2015)
13. L.I. Mei, M. Guang-shu, L. Hua, S. Xiao-lin, T. Ying, H. Wen-kun, M. Jie, and J. Xi-liang. *J. Integr. Agric.* **18**, 607–617 (2019)
14. H. An-le, L. Jia, W. Xin-hua, Z. Quan-guo, S. Wei, and C. Jie. *J. of Integr. Agric.* **18**, 599–606 (2019)
15. D. Murtadho, L. Setyobudi, and N. Aini. *Buana Sains* **16**, 143–150, (2016)
16. G. Fauza, Y. Amer, S.H. Lee, and H. Prasetyo. *Int. J. Prod. Econ.* **182**, 409–417 (2016)
17. S. Zeilinger, S. Gruber, R. Bansal , and P.K. Mukherjee . *Fungal Biol. Rev.* **30**(2): 74– 90 (2016)
18. S. Yuan, M. Li , Z. Fang, Y. Liu, W. Shi, B. Pan, K. Wu, J. Shi, B. Shen, and Q. Shen. *Biol. Control* **92**, 164–171 (2016)
19. K. Saravanakumar, C. Yu, K. Dou, M. Wang, Y. Li, and J. Chen. *Biol. Control* **94**, 37–46 (2016)
20. L. Zhao, Y. Xu, and X. Lai. Braz. *J. Microbiol.* **49**, 269–278 (2018)
21. D. Liu, K. Li, J. Hu, W. Wang, X. Liu, and Z. Gao. *Int. J. Mol. Sci.* **20**, 2908 (2019.)
22. M.R. Khan, F.A. Mohidin, U. Khan, and F. Ahamad. *Biol. Control*, **101**, 159–168 (2016)
23. M. Massoumi, F.A. Krens, R.G.F. Visser, and G.-J.M. De Klerk. *Plant Cell, Tissue Organ Cult.* **130**, 531–541 (2017).