ISSN 0115-463x
IF (2016) - 0.115

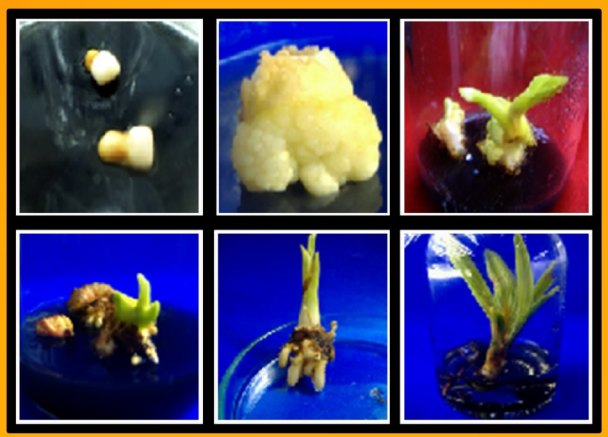
THE PHILIPPINE JOURNAL OF CROP SCIENCE

Published by the Crop Science Society of the Philippines
& The Federation of Crop Science Societies of the Philippines

Volume 41 No. 1 Special Issue June 2016

"... that whoever could make two ears of corn, or two blades of grass, to grow on a spot of ground where one grew before, would deserve better of mankind and do more essential service to his country..."

-- Jonathan Swift



CONTENTS:

PJCS is an ISI journal listed in the 15th of January 2007 'CABI Full Text Journals' accepted. Check url:*http://bg.au.poznan.pl/dzialy/download/CABI%20Full%20Text%20journals.pdf*. PJCS is being indexed and abstracted in Science Citation Index Expanded (Sci Research) and Journal Citation Reports/Science Edition.

THE PHILIPPINE JOURNAL OF
CROP SCIENCE

Aim and Scope. The Philippine Journal of Crop Science (PJCS, abbreviated as Philipp J Crop Sci) publishes original papers on all aspects of crop science and technology, be it in research, instruction, training or extension. Manuscript may be a research report, review, lecture, or policy statement. This reflects the perspective of its publisher, the Crop Science Society of the Philippines (CSSP), when it was founded in 1970 as expressed in the first divisions of CSSP: Crop Production and Management, Processing and Marketing, and Teaching and Extension, a similar outlook in the CSSP: Declaration of Faith. Since 2000, the journal is being co-published by the Federation of Crop Science Societies of the Philippines.

Abstracting and Indexing Coverage. The PJCS is found in the pages of Agridex, Biological Abstracts, Current Advances in Ecological Sciences, Current Awareness in Biological Science, Field Crop Abstracts, Herbage Abstracts, Plant Breeding Abstracts, Weed Abstracts, Horticultural Abstracts, Tropag and Rural Database or the Agricultural and Environment for Developing Regions, WinSPIR 2.0 Plant gene CD, and CAB International. Since January 2007, the journal has been selected for coverage in Thomson Reuters products and custom information services beginning with Vol. 32 (1) 2007, and indexed and abstracted in the Science Citation Index (SciSearch®) and the Journal Citation Reports/Science Edition.

Subscription Information. The journal comes out 3 times a year: April, August and December. International Subscription per year inclusive of shipping: US$120 corporate, US$120 libraries and public institutions, US$50 professional non-FCSSP members. Local subscription: PhP4,300 per year and PhP1500 per issue inclusive of shipping. Copies are sent by air mail to subscribers outside the Philippines. Subscription requests should be accompanied by either cheque (with Manila clearing in the Philippines) or Postal Money Order, issued in the name of **Crop Science Society of the Philippines**. Inquiries about subscription can be sent to: pjcs.uplb@up.edu.ph.

Address. All business communications, subscription orders and payments should be addressed to: **The Philippine Journal of Crop Science, Crop Science Society of the Philippines, PO Box 165, 4031 College, Laguna, Philippines**.

Contact. The CSSP can be reached at http://www.cssp.org.ph, while the Editor in Chief at pjcs.uplb@up.edu.ph or 6349-536-2217/2468.

Crop Science Society of the Philippines Inc.
University of the Philippines Los Baños
4031 College, Laguna

MESSAGE FROM THE CSSP PRESIDENT

It is with great pride to always reminisce the good track record of the Crop Science Society of the Philippines (CSSP) in supporting our members towards professional development in the field of crop science, through continuous sharing of tangible technical results. We therefore continue to elevate the standards of crop scientist to be at par (or even better) with our ASEAN and global neighbors. CSSP will also not be as it is, if not for, among others, the continuing success of Philippine Journal of Crop Science (PJCS), the society's journal or testimony of continuous dissemination of outstanding scientific and technical advancements in the field of crop science.

I take pride in the success of PJCS through quality papers being published, the competency of its editors and reviewers, as well as the professionalism of its staff. This is proven by the fact that its impact on readership resulted in increase in paper submission (both local and international submissions) and subscription. Moreover, last year, published papers in PJCS was well-recognized by the NAST. In order to further reach out, PJCS is venturing into increased presence in the digital world although online system of abstract request is already operational. Thanks to Dr. Rhodora Romero-Aldemita, our always dependable Editor-in-chief and her editorial staff. But PJCS will not rest in its laurels. It will continue to strive to be better and will continue to find ways to best serve CSSP's membership. This way, it will also seek to serve the agriculture profession and community and therefore the society-at-large.

May I invite all crop scientists to support our programs through the following: 1) reach out to our colleagues and friends in agriculture and crop sciences to become members; 2) sustain PJCS through submission of quality papers for publication, as wll as annual subscription; 3) encourage members to continue promote PJCS through their mother institutions, as well as through the network of like-minded sector; and 4) participate in the CSSP events such as the 4th National Convention of the Philippine Association of Agriculturist, Inc. (PAA) on Nov 13-18, 2016, hosted by CSSP in Grand Men Seng Hotel, Davao City, and the conference of the Federation of Crop Science Societies of the Philippines (FCSSP) in the second quarter of 2017.

To the BOT of CSSP, FCSSP and PAA, as well as our dear friends and supporters, I would like to thank you for this opportunity to meaningfully serve the society and membership in the coming months.

More power to all of us.

ARTHUR R. BARIA
President
CSSP, 2016-2017

Philippine Journal of Crop Science (PJCS) Special Issue 2016 41(1):1-7

Zygotic Embryo Excision and Somatic Embryogenesis Propagation of Kopyor Coconut

Sukendah[1*], Makhziah[1] and Witjaksono[2]

[1]University of Pembangunan Nasional "Veteran" Jawa Timur, Surabaya, East Java, Indonesia; [2]Biotechnology Research Center, The Indonesian Institute of Sciences, Cibinong, Indonesia.
[*]Corresponding author, sukendah@upnjatim.ac.id

Propagation of kopyor coconut can be done only by *in vitro* culture. However, the available *in vitro* methods produce only one plantlet per zygotic embryo cultured. This paper discusses two *in vitro* methods to increase production of plantlets and seedlings of kopyor coconut, (1) the zygotic embryo excision and (2) somatic embryogenesis. Half of the embryonic explants were excised from freshly isolated embryo of the kopyor coconut fruit and inoculated in Eeuwens solid medium containing 60 g L^{-1} sucrose, 2.5 g L^{-1} activated charcoal, and 2.5-7.5 mg L^{-1} BAP. Embryogenic calli were induced from longitudinally or horizontally excised zygotic embryo explants and cultured on Eeuwens media containing 2,4-D and organic addenda. Observation was done on the percentage of regenerated plantlet, percentage of embryogenic calli, number of somatic embryos, plantlet formation, and morphological analysis. The halved embryo explants regenerated plantlet and shoot up to 83%, while the halved germinating embryo regenerated plantlet up to 100%. BAP improved shoot growth but not root growth and the best was 5 mg L^{-1}. For somatic embryogensis, the addition of 2,4-D at 50-125 µM was required for induction of embryogenic culture. Addition of organic addenda such as casein hydrolysate, liquid coconut endosperm, and especially amino acid improved the induction of embryogenic culture. Morphological study demonstrated that somatic embryogenesis and the subsequent development of the somatic embryos were accompanied by the development of abnormal structures such as rootless shoot, shoot less root, multiple shoots, and non-developed structure. Nonetheless, plant has been regenerated from this process and in that case somatic embryos resembled the zygotic counterparts.

Keywords: Callus induction, *Cocos nucifera*, *in vitro*, micropropagation, somatic embryo

INTRODUCTION

Kopyor coconut, a naturally occurring coconut mutant which produces a soft and broken endosperm, is very rare in Indonesia. The tree is only found in Java and Sumatra Island. Unlike normal coconut, kopyor coconut has endosperm that fills the entire cavity of the nut. The water is not as much as the normal coconut, sometimes none. These characters make kopyor coconut interesting to develop and further enhance. In Indonesia, kopyor coconut is an expensive delicacy and its planting material alone is highly priced.

Kopyor coconut or macapuno in Philippines is a variant of coconut (Foale 2005) and resulted from natural mutation (Samonthe et al. 1989). Farmers usually obtain kopyor coconut from normal coconut seedlings. By this method and depending on where they are planted, the percentage of kopyor nuts produced is very low, only 1-2 fruits per bunch (del Rosario 1998). To increase the production of kopyor nuts per bunch to 100%, this can be done *in vitro* through embryo rescue. Although this technique can produce 100% kopyor nuts, the production of seedling is very low, with only one seedling from one cultured embryo explant. Therefore an alternative *in vitro* method should be undertaken to multiply plantlet rate to increase the number of seedlings produced. Development of a reliable clonal propagation method such as excised embryo explants and somatic embryogenesis would provide a rapid multiplication for kopyor coconut. Somatic embryogenesis technology has been used for mass propagation in oil palm (Chemalee and Te-Chato 2008: Muniran et al. 2008: Padua et al. 2013) and date palm (Esraghi et al. 2005).

Very few or limited research has been reported yet on *in vitro* propagation of kopyor coconut through excision of zygotic embryo. Meanwhile, propagation through somatic embryo may be patterned from the somatic embryogenesis of normal coconut. Some works on somatic embryogenesis of normal coconut have been carried out. Somatic embryo of normal coconut can be induced from callus (Monfort 1985; Blake 1990; Verdeil et al. 1992; Samosir et al. 1998). According to previous reports, callus could be induced from different sources of explants such as inflorescence and leaf (Blake 1990; Verdeil et al. 1992), plumule (Hornung 1995; Chan et al. 1997), and embryo (Karunaratne et al. 1989; Samosir et al. 1998). The recent works on improving of somatic embryo formation has been carried out from the callus of plumular (Fernando et al. 2004; Saenz et al. 2005; Perez-Nunez et al. 2006), immature zygotic embryo (Adkins et al. 2005). Sukamto (2011) tried to induce callus from the endosperm of normal coconut, even though many of them were browning.

Since kopyor coconut does not have masive and compact endosperm, the formation of callus can not be induced from this part. The possible source of explants in kopyor coconut can only come from the zygotic embryo. Although kopyor coconut contains an apparently normal embryo, we dont know yet its ability to form callus. Thus, the objective of this study was to examine the formation of kopyor seedling from excised zygotic embryo of kopyor coconut and its ability to form callus and somatic embryos to produce kopyor planlets.

MATERIALS AND METHODS

Plant materials

Zygotic embryos were used as source of explants. The embryos were excised from 11-12 month-old kopyor dwarf coconut fruits, collected from Jember, East Java, Indonesia. The embryo that is still enclosed with endosperm was sterilized in 1.0% sodium hypochlorite (NaOCl) for 10 min and rinsed three times with distilled water. They were then excised from the endosperm and sterilized using 0.5% NaOCl for 5 min, and washed three times with distilled water. They were placed in test tubes containing 10 mL liquid Eeuwens medium (Eeuwens 1976) and incubated in the culture room without light at 25 ± 2°C. The embryos that germinated, indicated by plumule or radical growth, were selected as explants.

Formation of plantlets from excised zygotic embryo explants

The haustorium of kopyor coconut embryo was discarded. Embryo without haustorium was sliced longitudinally into 2 pieces. Its embryo portion was cultured in the Eeuwens solid media. Media consisted of 2.5 g L^{-1} activated carbon, 60 g L^{-1} sucrose, 7 g L^{-1} agar, 2.5 mg L^{-1} 2,4-D and different concentrations of BAP (0, 2.5, 5.0, 7.5 mg L^{-1}). Every culture bottle was planted with 2 pieces of sliced embryo derived from one embryo. Cultures were arranged in CRD and maintained under dark condition at 25°C for 2 mo. After shoots and/or roots developed, the plantlet was subcultured to Eeuwens media containing BAP (2.5, 5.0, 7.5 mg L^{-1}), 2.5 g L^{-1} activated carbon, 60 g L^{-1} sucrose, and 7 g L^{-1} agar. Cultures were transferred to the light room with 16 h photoperiod. Subculture was done every 2 mo. Explants that grew into root were discarded while planlet without root were induced with IBA.

Formation of embryogenic callus

Callus induction. The germinated embryo was sliced into two parts after discarding the haustorium. The embryo was sliced longitudinally into 2, 4, 8 pc and cultured in the basal Eeuwens (Eeuwens 1976) medium containing 2,4-D (50, 75, 100, 125 µM), 2.5 g L^{-1} activated carbon, 60 g L^{-1} sucrose, 7 g L^{-1} agar and amino acid. The pH of the medium was adjusted to 5.8 before autoclaving for 20 min at 121°C. The experiment was arranged in CRD with three replications and each replication consisted of 15 samples. The cultures were incubated in the dark room at 25 ± 2°C and subcultured every month.

Callus proliferation. To multiply the embryogenic callus, callus culture was transferred into a new Eeuwens medium with decreasing level of 2,4-D. The level of 2,4-d was gradually reduced to half its previous level starting on the second transfer. Transfers were carried out every 4 wk. Embryogenic callus was maintained in darkness at 25 ± 2°C for 2-3 mo before transferring to the induction medium of somatic embryogenesis.

Formation of somatic embryo and germination

Nodular calluses from callus induction media containing 2,4-D were transferred into media consisting of 10 mg L^{-1} BAP, 10 mg L^{-1} BAP + 2.5 mg L^{-1} 2,4-D, 10 mg L^{-1} BAP + 5.0 mg L^{-1} 2,4-D. Media contained 60 g L^{-1} sucrose, 2.5 g L^{-1} activated carbon, 7 g L^{-1} agar to induce somatic embryogenesis. The pH of medium was adjusted to 5.8 before autoclaving for 20 min at 121°C. The experiment was arranged in CRD with three replications and every replication consisted of 15 samples. Cultures were transferred to fresh media every 4 wk. These were incubated at 25±2°C in a light room from cool white florescent lamps with 8 hr photoperiod.

The mature embryos were transferred to the media containing ABA (0, 25, 50 µM) suplemented by 60 g L^{-1} sucrose, 2.5 g L^{-1} activated carbon, 150 mL L^{-1} coconut water, and 7 g L^{-1} agar. Cultures were maintained in a light condition with 8 hr photoperiod. Somatic embryos that have changed in morphology were transferred to Eeuwens free hormone media to further develop the structures.

Morphological analysis

Callus was examined morphologically using a digital microscope NTSC System-DC2-456 type connected to the computer. The callus was observed at every phase of its development. Observation was further done on early formation of somatic embryo in the globular, mature, and germination phase.

RESULTS AND DISCUSSION

Formation of planlets from excised zygotic embryo explants

Kopyor coconut has no branch originating from the lateral bud development. However, the splitting technique with apical meristem in the embryo, turns out that the parts of their embryo can grow. More than 80% of the excised embryo explants survived on medium with 2.5 mg L^{-1} BAP (Table 1). The percentage of growing excised embryo decreased with increasing concentration of BAP. At a concentration of 7.5 mg L^{-1} only 58% of excised embryo was successfully grown. Without exogenous BAP, the excised embryo could still grow but very low, only 37.5% (Table 1). BAP increased the growth of explants more than two folds. However the sole addition of BAP into the media could not be done.

Table 1. Percentage of excised embryo explants that developed and formed into either shoots or roots or both shoot and roots (complete plantlet) at different concentrations of BAP

Concentration of BAP (mg L^{-1})	Percentage of explant grown	Percentage of explant that formed into shoot and root	Percentage of explant that developed into shoot	Percentage of explant that developed into root
0.00	37.50	0.00	25.00	12.50
2.50	83.33	8.33	62.50	12.50
5.00	70.83	0.00	50.00	20.83
7.50	58.33	8.33	37.50	12.50

BAP still need another growth regulator such as auxin (2,4-D) to increase the percentage of growing explants. If the BAP is provided without 2,4-D, the excised explants were difficult to grow and showed no response.

Not all the excised embryos could grow and become plantlets. About 20-40% of the excised embryo did not show any response probably because it has no shoot or root meristem. The excised embryo started to grow 5-7 d after culture on solid media (Figure 1C) and shoots and roots emerged after 3-4 wk. At 5-6 wk after culture, the excised embryo grew with white bud colour (no chlorophyll) (Figure 1D) and began to form chlorophyll (Figure 1E) after transferring in the light room. Plantlets started to form a new leaf after 2-3 months (Figure 1F) and turned into perfect plantlets.

Formation of embryogenic callus

Clonal propagation of kopyor coconut through somatic embryogenesis process has been successfully established, starting with callus induction. Callus of kopyor coconut could be induced from explants of zygotic embryo slices in the Eeuwens medium containing 2,4-D. Explants started to enlarge (Figure 2A) after 2-3 wk of culture and formed callus (Figure 2B) at 4-5 wk of culture. The callus continued growing and showed active cell division and proliferation leading to nodular callus formation. Kopyor coconut callus formation required synthetic auxin such as 2,4-D. In this study explants that were not given 2,4-D could not form callus in both intact and excised embryos. Growth regulator 2,4-D is often used to induce embryogenic callus in many palm trees (Eke et al. 2005; Fernando et al. 2011). It can also initiate the activation of genes for differentiation and enhance embryogenic cells through repeatitive cell division (Litz and Gray 1992).

However, callus formation was not influenced by the amount of 2,4-D added to the culture media. The effect is more on the development of fresh weight of callus. At 100 µM 2,4-D, callus weight was significantly higher than other concentrations (Table 2). Concentrations between 50-125 µM could induce the formation of kopyor coconut callus but differences were not significant. The average callus formation was above 60% at any level of 2,4-D (Table 2). At 2,4-D concentration above 125 µM, explant becomes brown and under 50 µM explant becomes swollen but do not form callus. In normal coconut, the optimum concentration of 2,4-D for callus induction was 100 µM (Chan et al. 1998) and 125 µM (Samosir et al.1998). Usually callus formation of normal coconut is

Table 2. Effect of longitudinally excised mode in kopyor coconut callus formation using Eeuwens media and at various concentrations of 2,4-D

Treatment	Percentage of embryogenic callus formed[1]	Callus diameter (cm)	Fresh weight of callus (g)	Number of embryos[2]/ inoculum
Excising mode:				
L-2 (Split 2)	90.97 a	1.1667	0.4980 b	17.0
L-4 (Split 4)	58.34 b	1.0583	0.7630 a	16.3
L-8 (Split 8)	60.43 b	0.9167	0.4728 b	11.0
Concentration of 2,4-D:				
50 µM	62.97	0.8333	0.5155 b	13.7
75 µM	78.70	1.1333	0.4243 b	13.2
100 µM	63.89	1.0222	0.8303 a	15.0
125 µM	74.09	1.2000	0.5415 b	16.0

[1]Means followed by the same letter within a column are not significantly different at 5% DMRT; [2]Embryo at early globular stage

observed in the presence of a high level of 2,4-D which results in the development of proembryoids (Blake 1990). However, high level of 2,4-D caused the explants of kopyor coconut to become necrotic. Although 2,4-D is required to stimulate callus formation, excessive levels tend to induce browning and inhibit growth of cultures (Ebert and Taylor 1990).

Kopyor coconut embryogenic callus mass developed into an individual proembryo after it is transferred to media containing less 2,4-D and added with BAP. Dussert et al. (1995) observed that embryogenic cells became separated from meristematic regions and developed into proembryo if the callus was transferred to a medium with low concentration of 2,4-D. Chan et al. (1998) also obtained coconut embryos by reducing the concentration of 2,4-D and the addition of 50 µM BAP. BAP has been widely used in the process of development and maturation of somatic embryos at various palm trees. In oil palm, 5 and 10 µM BAP are the best concentration for maturation of embryos and stimulate the development of shoot of the embryo (Duval et al. 1993). In date palm, development of somatic embryo is stimulated by a combination of 2,4-D with BAP (Eshraghi et al. 2005).

Beside from adding 2,4-D, callus formation of kopyor coconut need splitting of explants. Intact embryo could not form callus on all media containing 2,4-D (Table 2). Embryos were swollen and appeared to form calli but over time the embryo grew but without any callus formation. Excising allows meristematic cells of explants to come in direct contact with the medium

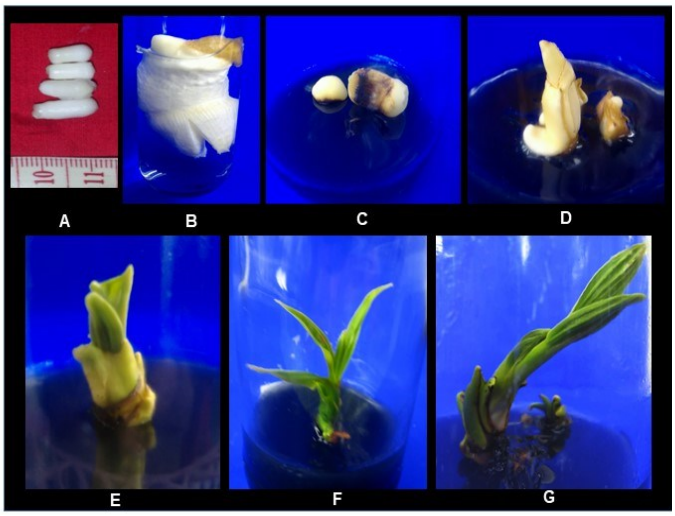


Figure 1. Regeneration process of kopyor coconut from excised zygotic embryo into a perfect plantlets on media containing BAP.

(A) Zygotic embryo explants; (B) Germinated embryo explants after 1 month culturing in Eeuwens liquid media, (C) Excised embryo explants grown on solid medium; (D) Excised embryo explants started to grow; (E) Excised embryo explants began to form green leaf; (F) Excised embryo explants developed to form a new leaf; (G) Normal plantlets regenerated from excised embryo

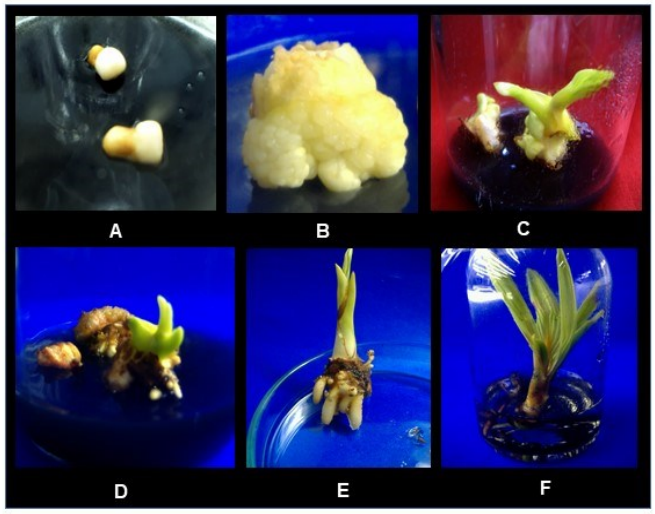


Figure 2. The morphology of kopyor coconut embryonic callus derived from excised zygotic embryo explants and plantlets at every stage of somatic embryogenesis.

(A) Excised zygotic embryo explants; (B) Embryogenic callus; (C) Somatic embryos germinated to form shoots within callus mass; (D) One of the embryos germinated within somatic embryo clump; (E) Plantlet derived from the germination of normal somatic embryo; (F) Plantlet from somatic embryo which have formed a perfect leaf

containing auxin or cytokinin, so the effect of growth regulator becomes more effective. Without excision, meristematic tissue of embryo explants is not exposed to the plant growth regulator.

Callus formation was also significantly affected by the mode of excision. Embryo excised into two parts formed callus 20% higher than that of the excised embryo of 4 or 8 parts (Table 2). The more excised embryos are, the percentage of callus formation tends to decrease. However, further callus development was not influenced by the size of the excised embryo. This is evident by the fresh weight of callus from embryos excised into 2 parts which was not much different with 8 excised embryos (Table 2).

Formation of somatic embryos and plantlets

The formation of somatic embryo appeared about 2-3 mo after culturing. Average number of somatic embryos on explant excised into 2 was not much different from the explant which was split into 4. As the number of excised embryo increases there was a tendency for the somatic embryos formed per explant to decrease. This was observed on embryo excised into 8 which produced an average of 11 somatic embryos per explant. Meanwhile, the average number of somatic embryos at high concentrations of 2,4-D was higher (100-125 µM) than at lower concentrations (50-75 µM) (Table 2).

After 2-3 wk, cultures transferred to the Eeuwens medium containing: a) 10 mg L^{-1} BAP, b) 10 mg L^{-1} BAP + 2.5 mg L^{-1} 2,4-D, and c) 10 mg L^{-1} BAP + 5 mg L^{-1} 2,4-D developed into mature somatic embryos. The individual somatic embryos showed further differentiation leading to germination or growth into certain structure. The number of somatic embryos on media with 10 mg L^{-1} BAP + 5 mg L^{-1}2,4-D, was two times more than the media containing 10 mg L^{-1} BAP + 2.5 mg L^{-1} 2,4-D. Likewise, media containing 10 mg L^{-1} BAP only have a slightly higher number of somatic embryos than media with 10 mg L^{-1} BAP + 2.5 mg L^{-1} 2,4-D (Table 3).

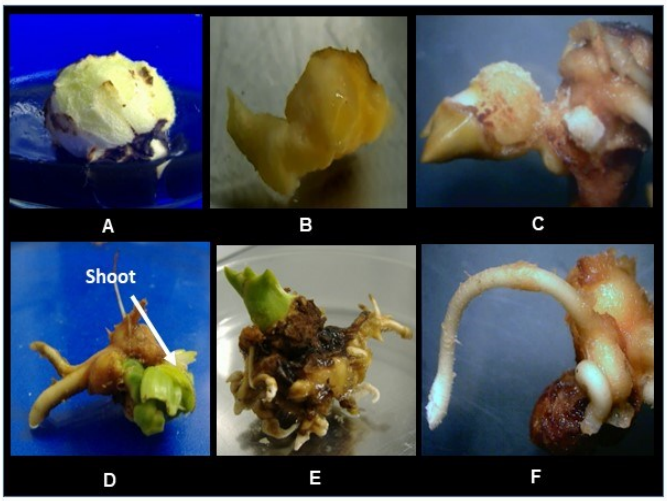


Figure 3. Morphology of non-embryogenic callus (A) and various abnormalities of kopyor coconut seedlings (B-F)

(A) non-embryogenic callus; (B) Seedling with shoot but without roots; (C) Embryos with formed shoot but without roots and roots growing from different embryo; (D) Seedling with multiple shoots; (E) Seedling with excessive root growth; (F) Roots without shoots

The percentage of complete plantlets (plantlet with shoot and root) produced on media containing 10 mg L^{-1} BAP + 5 mg L^{-1} 2,4-D was only 7%. If the 2,4-D

Table 3. The percentage of somatic embryos of kopyor coconut which developed into shoots, roots or plantlets in media containing 2,4-D and BAP

Growth regulator (mg L-1)	Number of embryos/inoculum	Percentage of germinated embryo	Percentage of embryo that developed into shoot	Percentage of embryo that developed into root	Percentage of embryo that developed into plantlet
10 mg L^{-1} BAP	8.6	70.14	18.75	40.27	11.11
10 mg L^{-1} BAP + 2.5 mg L^{-1} 2,4-D	7.4	85.71	14.28	49.97	25.38
10 mg L^{-1} BAP + 5.0 mg L^{-1} 2,4-D	14.8	69.05	8.33	53.57	7.14

Table 4. The percentage of somatic embryos of coconut kopyor which developed into shoots, roots, and plantlets on media with various concentrations of ABA

Concentration of ABA	Percentage of germinated embryo	Percentage of embryo that developed into shoot	Percentage of embryo that developed into root	Percentage of embryo that developed into plantlet
without ABA	62.49	10.49a	50.00	2.00 ab*
25 µM	66.65	5.81b	58.34	2.50 a
50 µM	72.30	1.30b	70.00	1.00 b

*Means followed by the same letter within a column are not significantly different at 5% DMRT

concentration was reduced to 2.5 mg L^{-1}, the percentage of complete plantlets that developed increased to 25%. Meanwhile, if 2,4-D is omitted or the media contained only 10 mg L^{-1} BAP, the plantlets obtained was 11%. Plantlets with shoots but without roots occurred more frequently in the media with 10 mg L^{-1} BAP than on media added with 2.5 or 5.0 mg L^{-1} 2,4-D (Table 3). For media that contained 10 mg L^{-1} BAP + 5 mg L^{-1} 2,4-D, despite producing more somatic embryos approximately 50% of somatic embryos grew into root structure, so the number of plantlets obtained was very few. Reducing the concentration of 2,4-D added to the BAP or not adding 2,4-D at all resulted to decreased formation of somatic embryos into roots. Somatic embryos that grow into root structure are usually discarded because these cannot be induced to form shoot.

Plant regeneration in some species through embryogenic callus were observed on the media containing 2,4-D and BAP. In *Sacchrum officinarum* the regeneration percentage was high on medium with 2.0 mg L^{-1} 2,4 D and 1.0 mg L^{-1} BAP (Naz et al. 2008) and in *Datura stramonium* higher frequency of plant regeneration of embryos were observed on the medium containing 9.04 mg L^{-1} 2,4-D and 13.32 mg L^{-1} BAP in addition with 3 mL L^{-1} coconut water (Sundar and Jawahar 2010).

Growth regulator ABA did not have much effect on the process of somatic embryo germination of kopyor coconut. Without the addition of ABA the percentage of coconut somatic embryos that grew into plantlets were not significantly different from treatment with ABA (Table 4). Addition of ABA stimulated the growth of the embryo into root structure. Without ABA the percentage of embryos that turned into shoot was higher. This implies that ABA is not required in the process of somatic embryo development of kopyor coconut. The use of high concentration of ABA (50 µM) also tended to increase the percentage of embryos that developed into root structure. This is in contrast to the results obtained by Samosir et al. (1998) and Fernando and Gamage (2000), which reported an enhanced formation and germination of somatic embryos of coconut with ABA treatment. Response of kopyor coconut to ABA is similar to the response observed in the embryo of avocado where the addition of ABA did not affect the production of somatic embryos, but enhances the formation of hyperhydric globular somatic embryos (Role-Quesada et al. 2004). In other crops such as date palm, high concentration of ABA reduced the number of embryos (Bawis et al. 2015) and inhibited the conversion of globular and heart embryos to shoot in tea plants (Ghanati and Ishka 2009).

Morphology of somatic embryo

The process of somatic embryo germination of kopyor coconut was not as simple as zygotic embryo. Somatic embryos cannot be germinated per individual embryo, although the embryos could be easily separated. In the germination medium, somatic embryos that were individually cultured turned brown resulting in death. Germination of somatic embryos occurred if they are still in the callus clump (Figure 2C and D). However, not all embryos in the callus can germinate and form complete plantlets (plantlets with shoot and root) (Figure 2E). Some somatic embryos transformed into an abnormal structure.

There were various forms of embryo that grow into abnormal structure. One form are embryos that are not equipped with the root apical meristem. When germinated, these embryos produce shoots without roots (Figure 3B). Nevertheless adventitious roots can be induced by treating with growth regulator like auxin. Another form are embryos where the shoots and roots

derived from different embryos were connected by a tissue (Figure 3C). Cotyledons or haustorium did not develop properly. Other abnormality were embryos that grew with many apical buds, resembling the form of bean sprouts which has more than one leaf bud (Figure 3D). There were also embryos that grow with excessive roots such that shoot growth slows down or part of shoots and roots were not proportional (Figure 3E). Last is the embryo that contained only the apical root and the haustorium, resulting in form without bud (Figure 3F).

One of the main problems in the process of somatic embryogenesis of kopyor coconut is the formation of normal plantlets/seedlings due to the incomplete process of embryogenesis, which is marked by numerous abnormal structure formation. Buffard-Morel et al. (1995) assumed that the deviation of coconut somatic embryo morphogenesis can be caused by hormone imbalance.

CONCLUSION

Clonal propagation of kopyor coconut has been established through excised zygotic embryos and somatic embryogenesis. Excised embryos grew into planlets in the medium containing BAP with low concentration of 2,4-D (2.5 mg L^{-1}). High concentration of BAP or without BAP tended to inhibit formation of plantlets. Propagation of kopyor coconut by somatic embryogenesis was done through callus induction in medium containing 2,4-D. This study reported high formation of somatic embryos on media with combinations of 10 mg L^{-1} BAP and 5 mg L^{-1} 2,4-D. However addition of 2,4-D in lower concentration increased the percentage of complete plantlets.

ACKNOWLEDGEMENT

We would like to thank the Directorate General of Research and Community Service, Ministry of Research, Technology and Higher Education, Republic of Indonesia for the grant which allowed us to conduct the research and to the staff of the Biotechnology Laboratorium for the technical assistance.

LITERATURE CITED

Adkins SW, Samosir YMS, Nikmatullah A, Ogle HJ. 2005. Coconut (*Cocos nucifera* L.) *in vitro* ecology: Modifications of headspace and medium additives can optimize somatic embryogenesis. In: Chang W-C, Drew R, editors. Biotechnology of tropical and subtropical species. Proc. the 2nd International symposium. Acta Hort 692, ISHS. p.21-31.

Bawis HA, Bujliah KA, Bu Ali MA, Bellaj ME, Abdallah AB. 2015. Effect of abscisic acid (ABA) on somatic embryogenesis in date palm (*Phoenix dactylifera* L). Int J Agri Sci Res. 4 (2): 031-034.

Blake J. 1990. Coconut (*Cocos nucifera* L.): Micropropagation. In: Biotechnology in agriculture and forestry: Legumes and oilseed crops I. Bajaj YPS, editor. Springer-Verlag, Berlin. p. 539-554.

Buffard-Morel J, Verdeil JL, Dussert S, Magnaval C, Huet C, Grosdemage F. 1995. Initiation of somatic embryogenesis in coconut (*Cocos nucifera* L.). In: Lethal Yellowing: Research and practical aspects. Oropeza C et al. editors. Kluwer Academic Pr. Netherland. p. 217-223.

Chan JL, Saenz L, Talavera C, Hornung R, Robert M, Oropeza C. 1998. Regeneration of coconut (*Cocos nucifera* L.) from plumule explants through somatic embryogenesis. Plant Cell Rep. 17:515-521.

Chemalee S, Te-Chato S. 2008. Induction of somatic embryogenesis and planlet regeneration from cultured zygotic embryo of oil palm. J Agric Tech. 4(2):137-146.

Del Rosario AG. 1998. Status of research on coconut embryo culture and acclimatization technique in UPLB. In: Batugal PA, Engelmann F, editors. Current state of the art and problems with *In vitro* Culture of coconut embryos. Proc. Symposium of the first workshop on embryo culture. Albay. p.12-16.

Dussert S, Verdeil JL, Buffard-Morel J. 1995. Specific nutrient uptake during initiation of somatic embryogenesis in coconut calluses. Plant Sci. 111:229-236.

Duval Y, Aberlenc F, de Touchet B. 1993. Use of embryogenic suspension for oil palm micropropagation. In: Recent development in oil palm tissue culture and biotechnology. Rao V et al., editors. Palm oil research institute of Malaysia, Kuala Lumpur. p. 38-47.

Ebert AW, Taylor QHF. 1990. Assessment of the changes of 2,4-dichlorophenoxyacetic acid concentration in plant tissue culture media in the presence of activated charcoal. Plant Cell Tiss Org Cult. 20:165-172.

Eeuwens CJ. 1976. Mineral requirements of cultural coconut tissue. Physiol Plant. 36:23-24.

Eke CR, Akomeah P, Asemota O. 2005. Somatic embryogenesis in date palm (*Phoenix dactylifera* L.) from apical meristem tissues from 'zebia' and 'loko' landraces. African J Biotech. 4 (3):244-246.

Eshraghi P, Zarghami R, Mirabdulbaghi M. 2005. Somatic embryogenesis in two Iranian date palm cultivars. African J Biotech. 4:1309-1312.

Fernando SC, Gamage CKA. 2000. Abscisic acid induced somatic embryogenesis in immature embryo explant of coconut (*Cocos nucifera* L.). Plant Sci. 151:193-198.

Fernando SC, Weerakoon LK, Gunathilake TR. 2004. Micropropagation of coconut through plumule culture. Cocos. 16: 01-10.

Fernando SC, Samarajeewa DADS, Liyanagedara LGNHK. 2011. Use of arabinogalactan proteins in coconut (*Cocos nucifera* L.) tissue culture: An alternative approach for improved tissue response. Cord. 27(2):9-16.

Foale M. 2005. An introduction to the coconut palm. In: Coconut Genetic Resources. Batugal P, Rao VR, Oliver J, editors. International plant genetic resources institute (ISBN 92-9043-629-8), IPGRI-APO. Malaysia.

Ghanati F, Ishka MR. 2009. Investigation on the interaction between abscisic acid (ABA) and excess benzyladenine (BA) on the formation of shoot in tissue culture of tea (*Camellia sinensis* L.). Int J Plant Prod. 3:07-14.

Hornung R. 1995. Micropropagation of *Cocos nucifera* L. from plumular tissues excised from mature zygotic embryos. Plantation Rech Develop. 212:38-41.

Karunaratne S, Periyapperuma K. 1989. Culture of immature embryos of coconut, *Cocos nucifera* L., callus proliferation and somatic embryogenesis. Plant Sci. 62:247-253.

Litz RE, Gray DJ. 1992. Organogenesis and somatic embryogenesis. In: Biotechnology of perennial fruit crops. Hammerschlag RE, editor. CAB International, Wallingword. p. 335-369.

Monforf S. 1985. Androgenesis of coconut, embryos from anther culture. Pflanzenzueeht. 94: 251-254.

Muniran F, Bhore SJ, Shah FH. 2008. Micropropagation of *Elaeis guineensis* Jacq. "Dura": comparison of three basal media for efficient regeneration. Indian J Exp Biol. 46:79-82.

Naz S, Ali A, Siddique A. 2008. Somatic embryogenesis and plantlet formation in different varieties of sugarcane (*Sacchrum officinarum* L.) Hsf-243 And Hsf-245. Sarhad J Agric. 24(4): 593-598.

Padua MS, Paiva LV, Labory CRG, Alves E, Stein VC. 2013. Induction and characterization of oil palm (Elaeis guineensi Jacq.) pro-embryogenic. An Acad Bras Sci. 85(4):1545-1556.

Perez-Nunez MT, Chan JL, Saenz L, Gonzales T, Verdeil JL, Oropeza C. 2006. Improved somatic embryogenesis from *Cocos nucifera* (L.) pumule explants. *In Vitro* Cell Dev Biol Plant. 42:37-43.

Role-Quesada R, Sanchez-Romero C, Barcelo-Munoz A, Pliego-Alfaro F. 2004. Factors affecting maturation of avocado somatic embryos. Scientia Hort. 102:61-73.

Sáenz L, Souza R, Chan JL, Azpeitia A, Oropeza C. 2005. ^{14}C-2,4 dichlorophenoxyacetic acid uptake and formation of embryogenic calli in coconut plumular explants cultured on activated charcoal-free media. Rev Fitotec Mex. 28(2): 151-159.

Samonthe LJ, Mendoza EMT, Ilag LL, Cruz ND, Ramirez DA. 1989. Galactomannan degrading enzyme in maturing normal and makapuno and germinating normal coconut endosperm. Phytochem. 28:2269-2273.

Samosir YMS, Godwin ID, Adkins SW. 1998. An improved protocol for somatic embryogenesis in coconut (*Cocos nucifera* L.). In: Dew RA, editor. Biotechnology Tropical & Subtropical Species. Proc. International Symposium. ISHS: Acta Hort. 461:467-474.

Sukamto. 2011. Effect of physiological age and growth regulators on callus browning of coconut endosperm *in vitro* culture. Biotropia. 18(1): 31-41.

Sundar AN, Jawahar M. 2010. Efficient plant regeneration via somatic embryogenesis from anthers of *Datura stramonium* L. J Agr Tech. 6(4): 741-745.

Verdeil JL, Huet C, Grosdemanges F, Rival A, Buffard-Morel J. 1992. Coconut somatic embryogenesis: obtention of several clone ramets. Oleagineux. 47: 465-468.

Philippine Journal of Crop Science (PJCS) Special Issue 2016 41(1):8-13

Antibiosis of Biological Agents *Streptomyces* sp., *Gliocladium* sp., and *Trichoderma harzianum* to *Fusarium Oxysporum* from East Java Indonesia

Penta Suryaminarsih[1]*, Kusriningrum[2], Ni'matuzahroh[3], Tini Surtiningsih[3] and Tri Mujoko[1], Sri Rahayuningtias[1]

[1]Department of Agritechnology, Agriculture Faculty, University of Pembangunan Nasional "Veteran" East Java, Idonesia; [2]Department of Veternary Medical, Airlangga University; [3]Department of Biology, Science and Technology Faculty, Airlangga University. *Corresponding author, penta_s@upnjatim.ac.id

This study was done to determine the species of biological agents *Streptomyces* sp. from Pare-Kediri tomato land and *Gliocladium* sp. from Food and Horticulture Crops Protection in Pandaan, and to know their antagonistic effects on *F. oxysporum* f.sp. *lycopersici* soil borne pathogens from Wajak village, East Java, Indonesia. Treatments were laid out in CRD with 4 replications. Biological agents were identified through its morphological characteristics and DNA sequensing. *Streptomyces* sp. was identified as *Streptomyces griseorubens* and *Gliocladium* sp. as *Gliocladium virens*. The results showed that *S. Griseurubens, G. virens* and *T. harzianum* were hiperparasites of *F. Oxysporum*. They can potentially compete with *F. oxysporum* for food and space in rhizosphere through the formation of pheripheral roots. The third mixture of these biological agents also produced antibiosis in the rhizosphere that could inhibit *F. oxysporum* f.sp. *lycopersici* growth.

Keywords: antagonism, biological agents, rhizosphere

INTRODUCTION

Fusarium oxysporum f.sp. *lycopersici* is a fungal pathogen of tomato that causes the plant to wilt. This fungus is a saprophyte that lives on the inorganic matter residue in the soil. Combination of several microbial soil saprophytes with multi-antagonistic mechanisms are more effective to pressure the population and activity of pathogens. Some biological agents are decomposers and growth hormone producers. Biological agent, *Streptomyces* sp., degrades carbon from crop residues and decomposes recalcitrant protein through proteolysis (Doi et al. 2008). *Streptomyces* sp. can also enhance plant height, fruit production, and flower count in tomato plants (Sastrahidayat 1994). On the other hand, *Trichoderma* sp. and *Gliocladium* sp., as biological agents, serve as biofertilizer that are packed in compost and help in the absorption of P and K elements and promote growth in plants. Furthermore, aside from promoting plant growth, *Gliocladium* sp., *Streptomyces* sp., and *Trichoderma harzianum*, are also effective biological agents to control pathogen population (Pal and Gardener 2006)

Several studies have shown the relationship between microbial pathogens and biological agents that support the process of biological control. *Trichoderma* sp. produces lytic enzymes that degrade chitin cell wall in mycoparasitic process and improve their own cell wall during division process (Chater and Chandra 2006) whereas *S. griseus* produces lytic enzymes that are capable of degrading fungal cell walls (Anitha and Rabeeth 2010). Moreover, pathogenic and non-pathogenic strains of *Fusarium* sp. that grow and develop on the same side of the roots of the host plant, compete for food (Olivain et al. 2006). *T. harzianum* produces indole-3-acetic acid (IAA) which induces the formation of lateral roots, increases root dry weight, induces plant resistance with xylanase inducer components, and colonizes plant roots (Gruber and Saiboth 2012; Semangoen 2000). The toxic metabolite produced by *Trichoderma virens* against *Phytium* sp. is gliovirin, while *T. harzianum* produces pyrone antibiotics against *Geomannomyces graminis* (Anitha and Rabeth 2010). The relationship of microbes in the soil can be described however, the process of recycling is very complex and involves biochemical reactions that the mechanism is still not understood (Benitez et al. 2004). On the other hand, fusarium wilt disease can be controlled using the combination of *Streptomyces* sp., *T. harzianum* and *Gliocladium* sp. The success of this control is determined by the antagonistic relationship between biological agents. *S. griseorubens, G. virens* and *T. harzianum,* as biological agents, are compatible to grow in Potato Dextrose Agar (PDA) medium and can form an association that does not harm each other or does not produce secondary metabolites that could inhibit the growth of each other. A mix of these two biological agents, *S. griseorubens* and *G. virens* (SG), *S. griseorubens* and *T. harzianum* (ST), and *G. virens* and *T. harzianum* (GT), and a mix of these three biological agents (SGT), could highly inhibit the development of the colony diameter of *F. Oxysporum,* as compared to a single biological agent *G. virens in vivo.* Furthermore, a mix of these two biological agents, *S*G and *S*T, as well as these three biological

agents, SGT, could inhibit the disease severity of tomato fusarium wilt caused by *T. oxysporum* f.sp. *lycopersici.* The aim of this study was to determine the species of biological agents *Streptomyces* sp. from Pare-Kediri tomatoes land, and *Gliocladium* sp., from Pandaan Food and Horticulture Crop Plants Protection, and to know their antagonistic effects on *F. oxysporum* f.sp. *lycopersici* soil borne pathogens from Wajak village, East Java, Indonesia.

MATERIALS AND METHODS

The materials for this research consisted of *Streptomyces* sp. isolates (from Pare-Kediri land tomatoes), *Gliocladium* sp., *Trichoderma harzianum* (from the collection of Food and Horticulture Crops Plants Protection in Pandaan), *F. oxysporum* from fusarium wilt diseases of tomato plants (from Wajak-Malang village), 80% sand soil, Potato Dextrose Agar medium (SAP Chemical), and Malt extract medium (Citroen).

The experiment was laid out in CRD with 5 treatments and 4 replications. The treatments were as follows: single biological agent (*Streptomyces* sp. (S), double biological agents (mixture of *Streptomyces* sp. with *Gliocladium* sp. (SG) and with *T. harzianum* (ST), three biological agents (mixture of *Streptomyces* sp., *Gliocladium* sp., and *T. harzianum* (SGT) and lastly, control (without biological agents).

Identification of Microorganisms

Biological agents were isolated using Dhingra and Sinclair (Singh et al. 2002) soil plating method wherein 1 g of soil in the chilli and tomato land was used to make the suspension by 10^{-4} dilution. *Streptomyces* sp. was isolated by preparing 1 mL suspension, which was taken asceptically and smeared, on a GNA medium. *T. harzianum* and *Gliocladium* sp. from PPFHC - Pandaan was isolated like *Streptomyces* but the isolation was done in a PDA medium. Biological agents obtained were then purified and propagated in petri dishes with the same PDA medium.

F. oxysporum was isolated by excision method. Parts of the stem of tomato plants infected with fusarium wilt were cleaned, sterilized with 70% alcohol, air dried, and sliced with a scalpel. The excised part was inoculated in PDA medium. Pathogenic fungi that have grown were isolated and purified. Pure *F. oxysporum* was then propagated in a PDA medium. On the other hand, *Streptomyces* sp. was identified in the microbiology laboratory of Tropical Diseases Center (TDC), Airlangga University, through 16S rRNA sequencing method. *Gliocladium* sp. was identified in the Laboratory Culture Collection of Institute Pertanian Bogor through 18S rRNA sequencing method. Colonies of *T. harzianum* were identified by macroscopic and microscopic observation in the Plant Health Laboratory of the National Development "Veteran" East Java University.

Antibiosis Test in the Rhizosphere

Antibiosis test was done on the 2nd, 4th, 6th, 8th, and 10th day after transplanting. One gram of soil was taken from the treatment and dissolved in a 10 mL sterile water for 1 min, centrifuged at 200 rpm, and then the 4 mL of this suspension was added to 46 mL of sterile water. The suspension obtained from this was then filtered with Whatman paper no. 44 and Zeis filter (5G) using vacuum pressure. The resulting filtrate was centrifuged at 150 rpm for 30 min. This solution containing antibiosis ingredients was stored in a refrigerator (4°C) for 24 hr. Antibiosis obtained was dripped on a filter paper disc (0.5 cm diameter Whatman paper) until saturated (0.55 cc), and then air dried. The paper discs containing antibiosis were inoculated on a PDA medium in petri dishes that had been inoculated with *F.oxysporum* spore suspension. Inhibition zones caused by filter paper disc on *F. oxysporum* was an indicator of inhibition. Further observations of the characteristics of the soil filtrate containing antibiosis were done in Biochemical Angler Laboratory using LC-MS/MS and analyzed by GC-MS, in Pharmaceutical Airlangga University.

RESULT

Identification of Biological Control Agents (BCAs).

Streptomyces sp. isolate had yellow, bright red, white like tissue cotton, unshiny colony, and a gram positive response. The hyphae morphology was 11 µm in diameter and was branched without septae. The spore was hyaline, long circular chain-shaped with 17.61 x 41.8 µm length, and 11.67-12.10 µm diameter. The DNA gen isolation result from supernatant and sediment from Polymerase Chain Reaction (PCR), showed the *Streptomyces* sp. on a gel electrophoresis in 1.2 kb area. This was similar with the *Streptomyces griseus* area where the primary common area was used for 16S rRNA (Figure 1).

Basic Local Alignment Tool (BLAST) analysis showed that for the majority of Actinomycetes, the result of the DNA sequensing was on the *Streptomyces griseorubens* species area, with a total number of 603, which is approximately similar to level 97% and level 98% with *Actinobacterium.*

On the other hand, *Gliocladium* sp. isolate had light green color, circular, solid, and soft middle surface, ratherthan white colony, and a grown up hyphae. This hyphae was branched, hyaline, and has a diameter of 28.50 µm. Conidium was 13.75-15.56 µm, circular, hyaline, branched, and stand on conidiophore. The result of *Gliocladium* sp. DNA sequensing on the agarose gel was showed on 1.5 kb area and was not different from the primary used *Gliocladium virens* (Figure 2). Phylogenetic analysis using BLAST submitted to Gene Bank, has identified a similarity level of 99.61% with a strain of *Trichoderma* sp. *INBio 3018F.*

Macroscopic observations on *T. harzianum* colonies appeared to be green, velvet shaped, and has a presence of circular zone. Conidia was round, hyaline

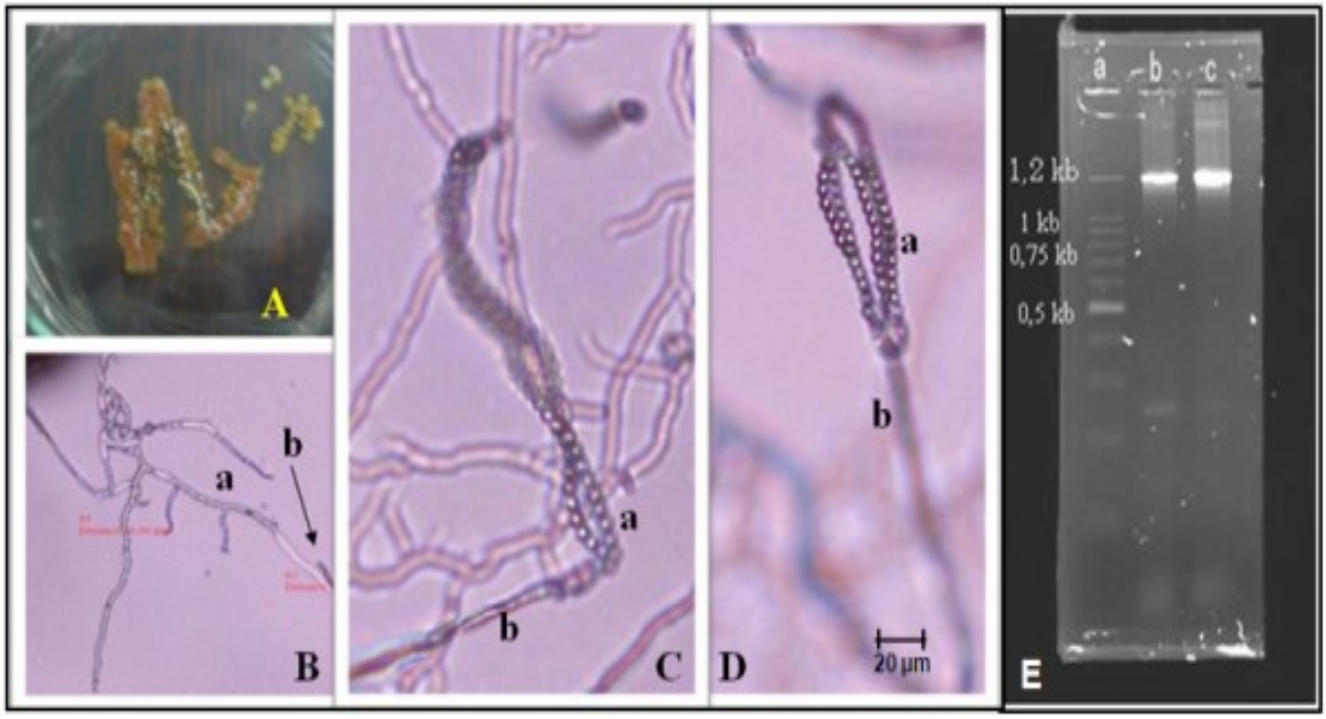


Figure 1. A. Colonies of Streptomyces sp. on PDA at 14 days; B. Morphology microscopic research of *Streptomyces* sp.; D. Circular spore microscopic research, magnified at 10 x 40. E.*Streptomyces s*p. DNA electroforesis result on gel agarose (TDC UNAIR)

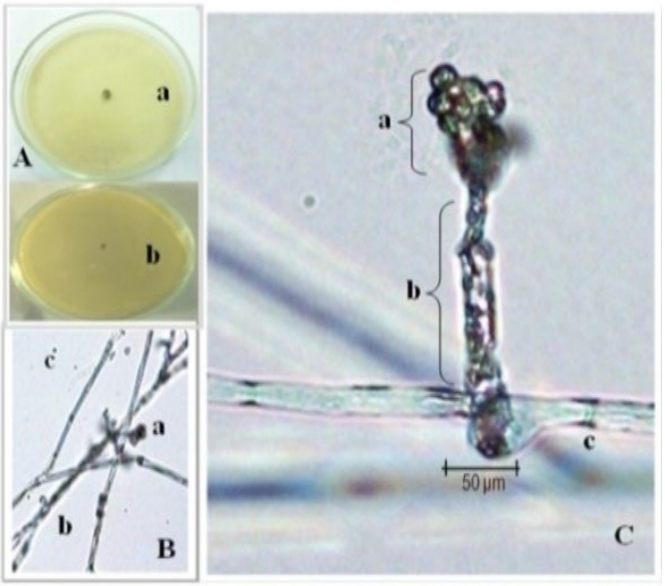


Figure 2. A. Colonies of *Gliocladium* sp. on PDA at 4 days; B. Microscopic observation of *Gliocladium* sp.; C. Microscopic observation of *Gliocladium* at a magnification of 10 X 40.

and has a measure of 14.65 µm. Filamentous hyphae with septae was branched, hyaline, and 35.75 µm in diameter (Figure 3).

Obstacle Results on the Antibiosis Soil Filtrate

The average inhibition zone from soil filtrate that consisted of single biological agent (*S. griseorubens*), mixed biological agents (*S. griseorubens, G. virens, T. harzianum*), and control towards the development of *F. Oxysporum,* were different starting from the 2nd, 4th, 6th, 8th and 10th d after inoculation. The average inhibition zone of soil filtrate consisting of *Streptomyces* sp., *Gliocladium* sp.*,* and *T. harzianum* mixtures was bigger than the soil filtrate with *S. griseurubens* only (Table 1).

The result of GC-MS analysis on the sand soil filtrate at 10th d after inoculation in the mass spectrum (m/z) was 243 g mol^{-1} and it was not streptomycin. On the other hand, LC-MS/MS analysis on the soil filtrate containing mixture of biological agents *S. griseurubens*, *T. Harzianum,* and *G. virens* at 10th d after inoculation with biological agents and *F. oxysporum* in the sand soil, showed that the retention peak of the filtrate was 2.64 min.

DISCUSSION

The result of the DNA sequensing of *Streptomyces* sp. that was uploaded to the world genbank had a 98% similar description with *Actinobacterium ZXY004* and *S. griseorubens strain 2418.* Moreover, based on the morphology of the colony character, the size and the spore shape of S*treptomyces* sp. isolate showed a similarity with the strain of *S. griseorubens* sp. by having a filament bacterium, small sprouted colony like cotton, and also produces *coccus* spore (Brown et al. 2012*).* Furthermore, *Actinobacterium ZXY* belongs to genus *Actynomycetes* and the another name of *Streptomyces griseorubens* was *Actinomyces griseorubens* (Cook and Baker 1974).

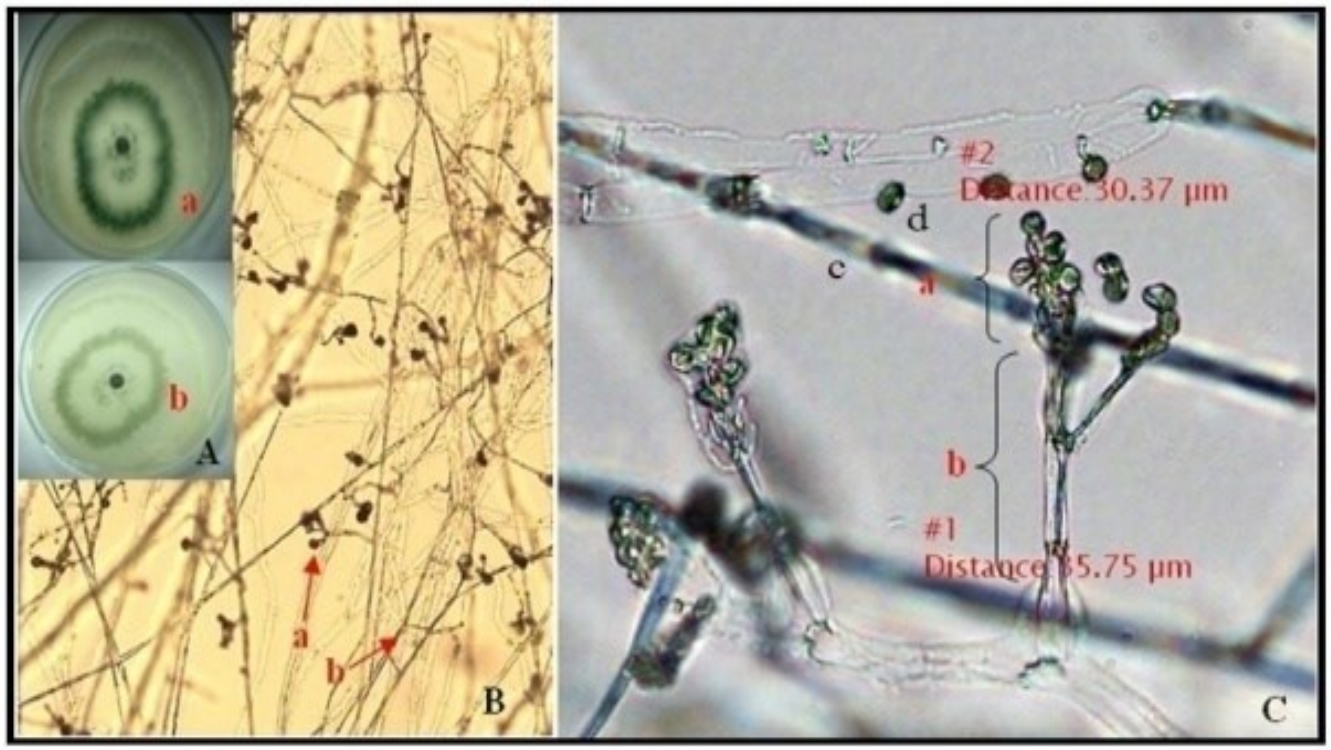


Figure 3. A. *Gliocladium* sp. colony on PDA medium at 4 days; B. *Gliocladium* sp. colony microscopic observation; C. *Gliocladium* sp. conidium mycroscopic observation, magnified at 10 x 40

Table 1. Average inhibition zone of antibiosis soil filtrate to *F. oxysporum*

Biological contol	Average inhibition zone									
	2 hsi		4 hsi		6 hsi		8 hsi		10 hsi	
	(cm)	Transf. $\sqrt{(x+0,5)}$	(cm)	Transf. $\sqrt{(x+0,5)}$	(cm)	Transf. $\sqrt{(x+0,5)}$	(cm)	Transf. $\sqrt{(x+0,5)}$	(cm)	Transf. $\sqrt{(x+0,5)}$
SGT	0,15	0.80 ^{b}	0,33	0.91 ^{a}	0,23	0.85 ^{a}	0,60	0,85 ^{a}	0,95	1,05 ^{a}
SG	0,00	0.71 ^{c}	0,00	0,71 ^{c}	0,00	0.71 ^{b}	0,10	0,78 ^{b}	0,40	0.78 ^{a}
ST	0,00	0.71 ^{c}	0, 08	0.76 ^{b}	0,10	0.79 ^{ab}	0,08	0.76 ^{b}	0,13	0.76 ^{a}
S	0,48	0,99 ^{a}	0,10	0.79 ^{b}	0,08	0.76 ^{b}	0.00	0.71 ^{b}	0,18	0.71 ^{a}
Control	0,00	0.71 ^{c}	0,00	0,71 ^{c}	0,00	0.71 ^{b}	0,00	0,71 ^{b}	0,00	0,71 ^{a}

BLAST analysis result on the DNA sequencing of *Gliocladium* sp. through 18S rRNA showed a 90% similarity with *Trichoderma* sp. The morphology of *Gliocladium* sp. was almost the same with *T. harzianum.* However, the uploaded result of BLAST analysis was similar with the pimary standard used, which was *Gliocladium virens.* In addition, Skreekanth et al. (2011) reported the differences on the colony surface in the PDA medium of *Gliocladium* sp. and *T. harzianum.*

On the other hand, as published by BPTPH Bogor, *Trichoderma harzianum* isolate from BPPHPTPH Pandaan had the same morphology features with isolate *T. Harzianum.* As these include light green to dark green colony, wool-like, produces asexual conidia with a globular shape, and the conodia forms like grapes and grows quickly (Akladious and Abbas 2012; Alam et al. 2003; Buchanan and Gibbon 1974).

Furthermore, filtrate from the soil observation showed an antibiosis that can inhibit *F. oxysporum.* The antibiosis on rhizosphere was from *G. virens* and *T. harzianum*. This statement was supported by the results from biological agents' population and the pathogenic fungus population. However, on the 30th d, the population of *S. griseorubens* was not found and the inhibition zone of soil filtrate consisting of *S. griseorubens* on the 8th and 10th d was 0 cm.

Biological agents, *T. harzianum* and *G. virens,* could grow and develop faster around the roots compared to *F. Oxysporum* on the 1st d after planting. The competition between biological agents and pathogenic fungus is mainly on the source of nutrition because on the 3rd day, *F. oxysporum* grew and developed on the same root as the biological agents. Some researches found that *T. harzianum* and *G. virens* are soil saprophytic fungi that can develop quickly. In 48 hr

both biological agents had formed colony and twisted the root and penetrated the intercellular root (Agrios 1994; Allexopoulus 1996; Singh et al. 2002). In vitro observation showed that both biological agents grow quickly and can obstruct the development of *F. oxysporum* and can form a colony in the roots of the plant (Cook and Baker 1974). The competition between pathogenic and non-pathogenic Fusarium is on the nutrients since both hyphae grow on the same side of the sprouted root (Olivain et al. 2006).

Analysis on the standard antibiotic streptomycin showed that the peak retention time can reach to 4.93 min. Analysis of this soil filtrate by GC-MSD showed that the peak of the TIC retention time was 5.33 min, whereas the standard solution of ethyl acetate was 1.37 min. Derivation of the soil filtrate retention peak time (5.33 min) showed a molecular weight of 243 g mol^{-1}, while the standard derivative used has different molecular weight. Moreover, comparison of similarities with the standard mass spectrum was 40% only. The moleculer weight of the antibiotic was 400 g mol^{-1} but *G. virens* produced *gliovirin* which generated some mass spectrum of 389, 388, 290, 270, 263, 248, 247, and 177 (m/z). Furthermore, the moleculer weight of this antibiosis detection by GC-MS was 243 g mol^{-1} and it was not streptomycin (LC-MS).

CONCLUSION

In this research *Streptomyces* sp. is *Streptomyces griseorubens* f.sp. *capsicum* and *Gliocladium* sp. is *Gliocladium virens*. Mix of *S. griseorubens* f.sp. *capsicum*, *G.virens* and *T. Harzianum* produced antibiosis to *Fusarium oxysporum F.sp lycopersici* in rhizosphere. The moleculer weight of this antibiosis detection by GC-MS was 243 g mol^{-1} and it was not streptomycin (LC-MS).

REFERENCES

Agrios GN. 1994. Plant pathology, 2nd ed. Academic press. New York, San Fransisco, London.

Akladious SA, Abbas SM. 2012. Application of *Trichoderma harziunum* T2as a biofertilizer supporting maize growth. African Journal of Biotechnology. 11(35):8672-8683.

Alam M, Satar A, Kumar S, Samad A, Dawam OP. 2003. *Streptomyces*strain with potential antimicrobial activity againts phytopatogenik fungi. United States Patent No US6,558,940 B2.

Alexopoulus CJCW, MimsBlackwell. 1996. Introductory ofmycology.4th ed. John wiley & Sons, Inc. New York Chichester Brisbane Toronto Singapore.

Anitha A, Rabeeth M. 2010[a]. Control of fusarium wilt tomato by bioformulation of *Streptomyces griseus* in green house condition. African Journal of Basic & Aplaid Sciences 1(1-2):9-14.

Anitha A, Rabeeth M, 2010. Degradation of fungal cell walls of phytopatogenic fungi by lytic enzyme of *Streptomyces griseus.* African Journal of Plant Science. 4(3):061-066.

Benitez T, Rincon AM, Limon MC, Codon AC. 2004. Biocontrol mechanism of strain of Trichoderma. International Microbiology. 7 (4):4-10.

Brown DW, Butchko RAE, Baker SE, Proctor RH. 2012. Phylogenomic and functional domain analysis of polyketide synthases in *Fusarium.*Fungal Biology. 116(2):318-331.

Buchanan RE, Gibbons NE. 1974. Bergey's manual of determinative bacteriology. The Williams & Wilkins comp. Baltimore. 1246 h

Cook JR, Baker KF. 1974. Biological control of plant pathogens.Freeman FE, company. San Francisco, 348 p.

Chater KF, Chandra G. 2006. The evolution of development in *Streptomyces* analysed by genome comparisons. Canadian Journal of Botany, Sep. 30(5):651-652.

Doi H, Kinoshita N, Oka T, Hui Z. 2008. Streptomyces strain that decomposes protein recalcitrant to proteolisis. Microbial Chemistry Research Foundation. US201230003717AI US Patent No. 7.344875.

Gruber S, Seiboth V. 2012. Self versus non-self: fungal cell wall degradation In Trichoderma. Microbiology 158:26-34

Kaewchai S, Soytong K, Hyde KD. 2009. Mycofungicides and fungal biofertilizers. Fungal Diversity 38: 25-50.

Olivain C, Humbert C, Nahalkova J, Fatehi J, Haridon FL,Alobouvete C. 2006.Colonization of tomato root by pathogenic and non patogenic *Fusarium oxysporums* strains inoculated together and separately into the soil. Applied and Enviromental Microbiology. 72(2):1523-1531.

Pal KK, Garderner BM. 2006. Biological control of pathogens. The plant health instructor. DOI: 10.1094/PIH-A-2006-111702.

Semangun.2000. Diseases of horticultural crops in indonesia. The fourth edition. Gajah Mada University Press. Well Bulak Yogyakarta.

Schirmbock M, Lorito M, Wang Y, Hayes CK, Atac IA, Scala F, Harman GE, Kubicek CP. 1994. Pararel formation and synergism of Hydrolytic enzymes and Peptaibol antibiotics, moleculer mechanisms involved in antagonistic action of *Trichoderma harzianum* againts phytopathogenic fungi. Applied and Enviromental Microbiology. 80(12):4364-4370.

Singh R, Singh BK, Upadhyay RS, Rai B, Lee YS. 2002. Biological control of Fusarium wilt disease of pigeonpea. Plant Pathology Journal, 18 (5) : 279-283.

Skreekanth D, Sushim GK, Syed A, Khan BM, Ahmad A. 2011. Moleculer and morphological characterization of taxol-producing endophytic fungus, *Gliocladium* sp., from Taxus baccata. Mycobiology. 39(3):151-157.

Suryaminarsih P, Mulyadi, 2003. Study of the influence of dose of *Trichoderma* sp. and the concentration of leaf fertilizer on the development of *Fusarium* wilt and yield of onion. Journal of the Faculty of Biology (in Indonesia). Biology 3:3.

Suryaminarsih P, Mujoko 2012. Compatibility mixture of biological agents *Streptomyces sp., Gliocladium sp., Trichoderma harzianum* in improving the growth of tomato plants infected with Fusarium wilt disease (In Indonesia). Proceedings of the National Seminar Perhorti.

Yedidia I, Benhamou N, Chet I. 1999. Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. Applied Enviroment Microbiology., 65(3): 1061-1070.

Philippine Journal of Crop Science (PJCS) Special Issue 2016 41(1):14-16

Formulation of Organic Fertilizer and Fluorescent Pseudomonad Pf-122 Isolate for Increased Wilt Resistance in Chili

Yenny Wuryandari*, Sri Wiyatiningsih and Maroeto

University of Pembangunan Nasional "Veteran" Jawa Timur, Surabaya, East Java, Indonesia,
*Corresponding author, yennywuryandari@upnjatim.ac.id

Chili is an important fruit vegetable crop with a good demand in the local and international market. However, its production is being hindered by diseases caused by *R. solanacearum* and *F. oxysporum* resulting to wilting of the plant. This research aimed to determine which form of Fluorescent Pseudomonad Pf-122 isolate with organic fertilizer could greatly enhance the resistance of chili to bacterial wilt. The study was laid out in CRD composed of three formulation treatments (pelleted, granulated and powdered organic fertilizer with Fluorescent Pseudomonad Pf-122 isolate) and control (organic fertilizer only) with three replications. During the incubation period, results revealed that the slowest incubation period for the disease manifestation was observed in pelleted and powdered form fertilizer (7 d) followed by granulated (6 d) and lastly by control (5 d). The development of disease in chili treated with powdered formulation showed the slowest rate among other formulations. On the other hand, as compared with the control, the powdered formulation suppressed the development of disease by 42.67% while the granulated and pelleted fertilizer suppressed by 34.67% and 28%, respectively. Thus, application of powdered form Fluorescent Pseudomonad Pf-122 isolate with organic fertilizer could fully enhance the resistance of chili to bacterial wilt.

Keywords: formula, granule, pellet, powder, pseudomonad fluorescent

INTRODUCTION

Chili (*Capsicum annuum* L.) is a fruit vegetable crop that has high economic potential as there is demand in the local and international market. However, chili production is being hindered by *Ralstonia solanacearum* and *Fusarium oxysporum* which cause the plant to wilt. The said diseases can cause significant yield losses in chili. To counteract the effects of the diseases, conventional farmers opted to use pesticides. The possibility of using bacteria as an alternative to pesticides should be explored because bacteria has the ability to induce systemic resistance by producing phytohormones, dissolving inorganic phosphate and increasing the bind of iron to siderophores (Podile and Kishore 2007; Haas and Defago 2005). Rhizobacteria, a known biological control agent, induces the production of secondary metabolites such as siderophores, antibiotics and hydrogen cyanide. It also offsets spur growth through auxin derivatives (Pinton et al. 2001). Some rhizobacteria that stimulate plant growth hormones are *Pseudomonas*, *Bacillus* and *Azobacter* (Brimecombe et al. 2001).

Pseudomonas suppresses the infestation of bacterial wilt in chili while *Pseudomonas flourescens* promotes resistance in tomato, potato, and eggplant. Recent studies have also revealed that on a green house scale, the powder formula with active ingredients of PF suppresses the most effective wilt disease, namely the 41.67 disease index (Wuryandari et al. 2015). This research aimed to determine which form of Fluorescent Pseudomonad Pf-122 isolate with organic fertilizer could greatly enhance the resistance of chili to bacterial wilt.

MATERIALS AND METHODS

The experiment was conducted in a chili plantation at Lebo, Sidoarjo, East Java from May 2015 to July 2015. The study was laid out in CRD and the treatments were replicated three times. The data gathered were subjected to ANOVA procedure using SPSS (Statistical Product and Service Solutions). The treatments for this study consisted of pellets, granules, powder and the control. The control was organic fertilizer only manufactured by Factory Mini UPN East Java. The organic fertilizer contained sugarcane waste, rock phosphate, guano and cow dung with a ratio of 3kg: 1kg: ¾ kg: ½ kg, respectively. The organic fertilizer was mixed with Fluorescent Pseudomonad Pf-122 isolate at a concentration of 10^{10} cfu mL^{-1} and then formed into pellets, granules and powder. Ten grams of the formulation were then placed in holes near the 8-wk-old chili plants.

The parameters observed in this experiment were the incubation period and disease severity. Incubation period corresponded to the number of days at which symptoms appeared from inoculation. Disease severity was assessed every 5 d and evaluated using the following scale: 0= no symptoms, 1= 1-10% wilted

leaves, 2= 11-30% wilted leaves, 3= 31-60% wilted leaves, 4= 61-99% wilted leaves, and 5= 100% wilted leaves.

The magnitude of the disease index was calculated based on the formula of Arwiyanto (1995):

where:

$$I = \frac{\sum_{i=0}^{k} k.nk}{ZxN} \times 100\%$$

I = disease index
k = scale or score
nk = number of plants symptomatic pain scale k (0, 1, 2, 3, 4, 5)
N = the total number of plants inoculated
Z = category of highest attack

RESULTS AND DISCUSSION

At the end of the observation period, disease index of the different forms of organic fertilizer with Fluorescent Pseudomonad Pf-122 isolate were significantly lower than that of the organic fertilizer alone (Table 1). This indicates that Fluorescent Pseudomonad Pf-122 isolate effectively suppressed the manifestation of bacterial wilt. On the other hand, the disease index of powder was significantly lower (13.33) compared to granule (21.33) and pellet (28.00).

In agreement with the results of disease index, the most effective formulations to delay the manifestation of bacterial wilt symptoms were powder and pellet. The Fluorescent Pseudomonad Pf-122 isolate with organic fertilizer in the forms of pellet and powder delayed the symptoms by 2 d compared to the control. However, any form of Fluorescent Pseudomonad Pf-122 isolate could significantly delay the disease infestation (Figure 1). More so, in a field condition, extraneous variable including other pathogenic activity may affect the population of Fluorescent Pseudomonad Pf-122 isolate. Thus, hindering its ability to inhibit the resistance of chili plant.

As seen in Figure 2, Fluorescent Pseudomonad Pf-122 isolate with organic fertilizer in powdered form suppressed the development of disease by 42.67% whereas the granulated and pelleted form were able to halt the development of disease by 34.67% and 28%, respectively. Based on the disease index gathered during the observation period, wilt disease development in plants treated with powder formulation showed the slowest progress of disease. It was evident that the application of powdered formula was the most influential in counteracting the wilt disease caused by *Fusarium* and *Ralstonia solanacearum*. The presence of biological agent (Fluorescent Pseudomonad Pf-122 isolate) in the formulation suppressed the development of pathogens that caused wilting. In powder formulation, Fluorescent Pseudomonad Pf-122 isolate was better developed

Table 1. Bacterial wilt severity of chili in response to different Fluorescent Pseudomonad Pf-122 isolate with organic fertilizer formulation

Formulation	Disease Index
Powder	13.33 a
Granule	21.33 b
Pellet	28.00 c
Control	56.00 d

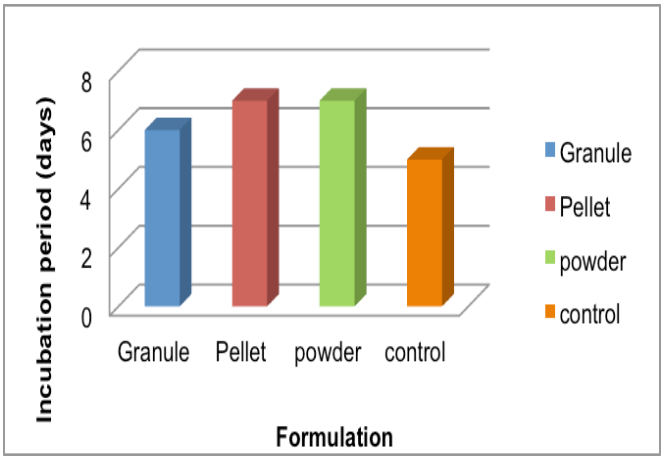


Figure 1. Incubation period of bacterial wilt of chili in response to different forms of organic fertilizer with Fluorescent Pseudomonad Pf-122 isolate

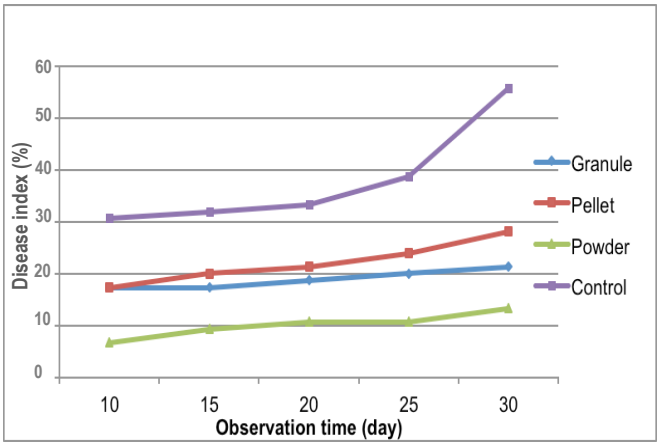


Figure 2. Disease development of bacterial wilt of chili with different forms of organic fertilizer containing Fluorescent Pseudomonad Pf-122 isolate

compared to other formulations. Moreover, the powdered formulation can be applied directly to the soil thus, the population of Fluorescent Pseudomonad Pf-122 isolate can grow faster than the other formulations. Furthermore, there are biological agents in the Fluorescent Pseudomonad Pf-122 isolate powdered formulation that can be developed due to high porosity conditions, unlike pellets and granules thus, having an adequate oxygen demand and higher bacterial population. This also confirms the findings of Palleroni (1984), that bacterium *Pseudomonas fluorescens* is an obligate aerobic bacteria.

Furthermore, increased surface area of the contact may have influenced the effectivity of Fluorescent Pseudomonad Pf-122 isolate. As observed, the less compact the Fluorescent Pseudomonad Pf-122 islolate in form, the lower the disease severity of bacterial wilt. This allows the Fluorescent Pseudomonad Pf-122 isolate population to be exposed to sufficient oxygen level thus, promoting propagation. This also justifies the findings of Palleroni (1984) that *Pseudomonas fluorescens* is an obligate aerobic bacteria. In addition, other bacteria that have potential phosphate solvent are *Pseudomonas* and *Bacillus*. Some saprophytic bacteria that act as biological control agents for bacterial wilt disease are *Pseudomonas fluorescens* in tomato, potato and eggplant, and *Pseudomonas* sp. in chilli (Saddler 2005). Moreover, bacteria have the capability to stimulate systemic resistance in plants to produce phytohormones, dissolve inorganic phosphate, and increase the binding of Fe with siderophores (Podile and Kishore 2007; Haas and Defago 2005).

CONCLUSION

The most effective formulation of Fluorescent Pseudomonad Pf-122 isolate with organic fertilizer to enhance the resistance of chili to bacterial wilt is the powdered form.

ACKNOWLEDGEMENT

The researchers would like to thank the Director General of Higher Education of the Republic of Indonesia for supporting this research through financial means and the Plant Health Laboratorium members for the technical works they have provided.

LITERATURE CITED

Beattie GA. 2007. Plant-associated bacteria: Survey, molecular phylogeny-genomics and recent advancesin: Plant-associated bacteria. Gnanamanickam SS. Editor. Springer The Netherlands. p. 1-56.

Brimecombe MJ, De Leij FA, Lynch JM, 2001. The effect of root exudates on rhizosphere microbiae populations. in: The rhizophere. biochemistry and organic substances at the soil-plant interface. Pinton R, Varanini Z, Nanipieri P, Dekker M. editors, Inc. New York – Basel. p. 95-140.

Haas D, Defago G. 2005. Biological Control of Soil Borne Pathogens by fluorescent *pseudomonads*. Nat Rev Microbial. 3:307-319.

Palleroni NJ. 1984. *Pseudomonadaceae*. In. Bergey's Manual of Systematic Bacteriology. Krieg NR,Holt JG. Editors. Baltimore: The Williams and Wilkins Co. p. 141-199.

Pinton R, Varanini Z, Nanipieri P. 2001. The rhizophere as a site of Biochemical Interactions Among Soil Components, Plants, and Microorganisms. In: The Rhizophere. Biochemistry and Organic Substances at the Soil-Plant Interface. Pinton R, Varanini Z, Nanipieri P, Dekker PM. Inc. New York–Basel. p. 1-18.

Podile AR, Kishore GK. 2007. Plant growth-promoting rhizobacteria.In: Plant-associated bacteria. rhizobacteria gnanamanickam SS. editor. Springer. The Netherlands. p. 195-230.

Saddler GS. 2005. Management of bacterial wilt disease. In:bacterial wilt disease and the *Ralstonia solanacearum* species complex. Allen C, Prior P, Hayward AC.editors. The American Phytopathological Society Press. p.121-132.

Wuryandari Y, Purnawati A, Siswanto. 2009. Viability of pseudomonads fluorescent Pf-122 in Multiple Formula. Periodic Conservation Biology Journal of Mathematics and Natural Sciences UNAIR .14 (2): p. 33-41

Wuryandari Y, Purnawati A, Siswanto.2011. Effect of formulation with Pseudomonad fluorescens Isolate Pf-122 for progress of tobacco bacterial wilt disease. In: Koentjoro Y. editor. The international seminar on natural resources, climate change, and food security in developing countries. ISBN 978-602-8915-93-9. p. 201-209.

Wuryandari Y, Wiyatiningsih S, Maroeto. 2015. Formula active ingredient in fluorescent pseudomonad and its effect on the development of wilt disease in chili. Journal of Tropical Plant Pests and Disease. 15 (1). p.89-94

Philippine Journal of Crop Science (PJCS) Special Issue 2016 41(1):17-21

Seeds and Agro-Industrial Wastes as a Medium for Growing Oyster Mushrooms (*Pleurotus ostreatus*) in the Nursery

Widiwurjani* and Guniarti

University of Pembangunan Nasional "Veteran" Jawa Timur, Surabaya, East Java, Indonesia.
*Corresponding author, widiwurjani@upnjatim.ac.id

This research aimed to identify the best substitute materials for oyster nursery medium and analyze the mycelial growth of mushrooms. The materials used for the major medium were seeds while for the starter medium were agro-industrial wastes. The experiment consisted of two factors. The first factor was corn seeds (major medium), kapok seeds, and *Leucaena* seeds. The second factor was agro-industrial wastes which consisted of three kinds viz. bran (major medium), soybean seed coat and tofu wastes. The results showed that both seeds and agro-industrial wastes were potential medium and can be used for growing oyster mushrooms. These responses were because seeds contain fiber and lignin and agro-industrial wastes contain essential nutrients required for the growth of the oyster mushrooms. Corn seeds were more effective in supporting the growth of mushrooms when mixed with any of the three agro-industrial wastes. On the other hand, kapok seeds were better than *Leucaena* seeds because it contains more fiber and carbohydrates. It also contains high nutritional value of about 32.7% protein and 16.7% fiber. For the industrial wastes, bran contains 10% carbohydrate, 7.5% protein, 2.25% fat and up to 7.5% variety of minerals (Nursiam 2012). Tofu waste contains enough protein to stimulate early mycelial growth. It contains about 414 kcal energy, 26.6 g protein, 41.3 g carbohydrates, 18.3 g fat, 19 mg Calcium (Ca), 29 mg Phosphorus (P) and 4 mg Iron (Fe). The medium made from kapok seeds and bran (T3L1) and kapok seeds and tofu wastes (T3L2) were able to support the mycelial growth with a speed of 0.39 cm d^{-1} and 0.36 cm d^{-1}, respectively. An increase in the weight of mycelium by 1.56 g d^{-1} and 1.93 g d^{-1} was observed in T3L1 and T3L2, respectively. Corn seeds can be substituted to *Leucaena* and kapok seeds as a growing medium for oyster mushrooms whereas bran can be substituted to tofu waste and soybean seed coat.

Keywords: agricultural waste, mycelial growth, nursery substrates, oyster mushroom

INTRODUCTION

One attribute of a good nursery is the capability to produce high quality oyster mushrooms that can adapt to a wide range of environmental conditions (Amni 2005). More so, one of the factors that can contribute to the success of the nursery is the choice of medium. Thus, nutrient availability in the raw material medium is important as it is critical for the cultivation and maintenance of oyster mushrooms. This has also something to do with the C and N ratio values, which are two equally important elements, responsible for the growth and development of oyster mushrooms. Moreover, there are two kinds of medium being used in the nursery, one is the major medium which serves as a supplier of lignin and second is the medium starter which supports the growth rate of the mycelium (Wuye 2013).

Other than sawdust, woods and seeds/grains can be used as medium for culturing and growing mycelium. According to Fithrawan (2010), these grains have high rate of success and are inexpensive and easy to produce. The commonly used grains are rice, corn and soybean flour. Moreover, the main advantage of grain is the high availability of nutrients that can be used for the growth of the mushroom. However, the drawback is the high risk of contamination.

The major and starter medium can be all made from seed grains such as corn, sorghum, soybean, rice and other material like sawdust. Hamdiyati (2012) explains that spawn F1 is the result of the generations of PDA seed. Furthermore, *Leucaena* and kapok seeds can be used as major medium which supply lignin to the culture and serve as a substitute for corn. On the other hand, starter medium can be made from selected agro-industrial wastes such as tofu waste and soybean seed coat as substitute for bran. All these materials are mixed together as one to form a rich nutrient-growing medium and be able to produce high quality oyster mushrooms.

This research focused on making a nursery medium from different grains and agro-industrial wastes. The study also aimed to get the best medium that would allow mycelial growth and produce high quality oyster mushrooms.

MATERIALS AND METHODS

Growing medium

The treatment consisted of two types of medium, the main medium and the starter medium. The main medium served as factor 1 which consisted of corn

seeds (major medium), kapok seeds, and *Leucaena* seeds. The starter medium served as factor 2 which consisted of bran (major medium), soybean seed coat and tofu wastes. The experiment was laid out in CRD and replicated three times.

Parameters Measured

Horizontal Mycelial Growth Speed (HMGS). The measurement for the HMGS was done using petri dishes. In the petri dish lid, labels and patterns of circles was made with the following radius: 1 cm, 2 cm, 3 cm, 4 cm, and 5 cm. Measurement of mycelial growth rate (cm d^{-1}) was done every time the mycelial growth reaches the boundary line of each circle. According to Christinawati (2003), HMGS can be computed using the formula:

$$\text{HMGS} = \frac{\text{size of mycelium in the petridish (cm)}}{\text{the time taken by mycelium to meet the size scale (day)}}$$

Vertical Mycelial Growth Speed (VMGS). The measurement for the VMGS was done using small bottles. The bottles were given a scale from the neck down to its base. Marking lines were made with scale ranging from 1cm to 25 cm with an interval of 1 cm. Measurement of mycelial growth rate (cm d^{-1}) was done every time the mycelial growth reaches the interval boundary lines. According to Christinawati (2003), VMGS can be computed using the formula:

$$\text{HMGS} = \frac{\text{size of mycelium in the bottle (cm)}}{\text{the time taken by mycelium to meet the size scale (day)}}$$

Mycelium and Seed Weight. The weight of mycel ium and seed at the end of its growth was calculated by getting the difference between the weight of the bottle (with the mycelium and seed) and the weight of the empty bottle used.

RESULTS AND DISUSSION

Mycelial Growth Speed

The observations on the mycelial growth rate of oyster mushroom are presented in Table 1. Results showed that mycelium can grow regardless of the medium used in the experiment. However, the growth speed was different among treatments. The combination of Kapok seed and bran (T3L1) and kapok seed and tofu waste (T3L2) did not show a significant difference in growth with the other medium. In contrast, all the medium made from corn seeds combined with all starter medium made from agro-industrial wastes gave the best result for mycelial growth (Figures 2 and 3).

The medium for growing oyster mushrooms in the nursery consisted of several materials. The materials that contain wood or lignin served as the main ingredient for the growth medium of mushrooms whereas the materials that are rich in nutrients served as a starter for the growth of molds. The use of corn kernels mixed with either of the three starter medium (T1L1, T1L2 and T1L3) gave the best result for the speed of weight growth. This is mainly because corn meal, according to Nursiam (2012), contains 73.7% carbohydrates, 9.2% protein, 1% Ca, 2.56% P, 0.24% Fe, and 0.0038% vitamin B1. Therefore, all these nutrients are good contributors for mycelial growth.

Table 1. Average mycelial growth speed of oyster mushrooms

Treatment	Average growth speed (cm d^{-1})		Ave
	horizontal	vertical	
T1L1 (maize + rice bran)	0.36 c	0.40ab	0.38 b
T1L2 (Corn + Tofu Pulp)	0.34 bc	0.42b	0.38 b
T1L3 (Corn + Soybean Seed Skin)	0.41 c	0.41ab	0.41 b
T2L1 (*Leucaena + bran)*	0.26 ab	0.33a	0.29 a
T2L2 (*Leucaena + Tofu Pulp)*	0.35 bc	0.36ab	0.36 a
T2L3 (*Leucaena + Soybean Seed Skin)*	0.23 a	0.38ab	0.31 a
T3L1 (Kapok + bran)	0.37 c	0.41ab	0.39 b
T3L2 (Kapok + Tofu Pulp)	0.36 c	0.37ab	0.37 ab
T3L3 (Kapok +Soybean Seed Skin)	0.24 a	0.34ab	0.29 a
LSD	0.09	0.08	0.08

*values with the same letter within a column are not significantly different at 5% LSD

The medium derived from the kapok seeds supplemented with a starter tofu waste, which is an agro-industrial waste (T3L2), was also able to provide mycelial growth. This, however, was not significantly different with that of the rice bran that has been used by farmers as a starter medium for growing oyster mushrooms. Moreover, *Leucaena* seeds were expected to give good mycelial growth due to its high protein content. Unfortunately, this was not achieved in the study. In a similar experiment conducted by Wuye (2013), the medium mixed with straw shoots, with a ratio of 2:1, had high protein content and yet the production of mushroom was still low. This suggests that the level of protein also affects the production of mushroom. When the protein content is too high, it could lead to a low production of mushrooms. Conversely, kapok seeds are way better than *Leucaena* seeds as it contain more fiber and carbohydrates. Kapok seeds have high nutritional value of about 32.7% crude protein and 16.7% crude fiber (Siregar 1994). Furthermore, Taufik (2005) said that the skin of the kapok seed is a waste obtained from stripping the seed prior to the extraction of oil and from the separation between the grain and kapok for furniture industry uses (mattresses). Kapok seeds also contain about 3-8% kapok limit in the form of cellulose

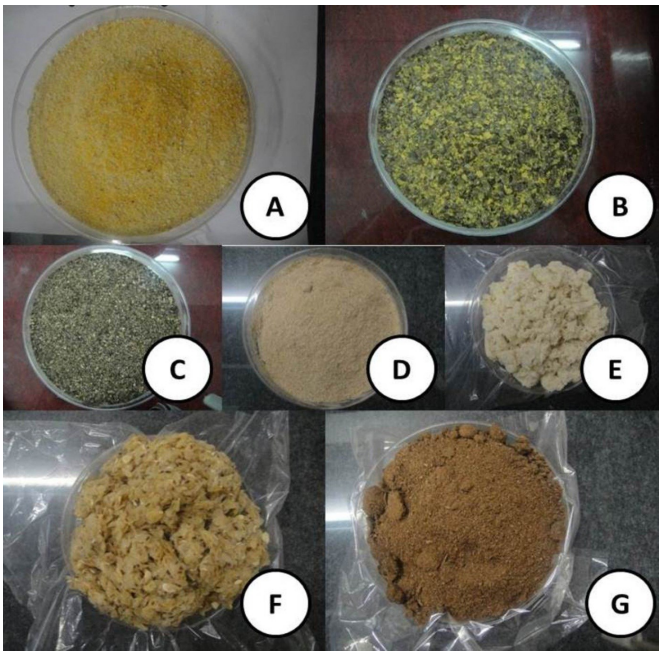


Figure 1. Agro-industrial waste materials tested as a medium for oyster mushroom nursery (A) Corn Seed (control); (B) *Leuceana* seed; (C) Kapok seed, (D) Bran (control); (E) Waste of tofu; (F) soybean seed coat; (G) Sawdust

that can easily be digested. In addition, the chemical content of kapok seeds are 91% dry matter, 4.1% crude protein, 42% TDN, 64% ADT, 90% NDF, 47.8% crude fiber, 1.7% crude fat and 2.8% ash.

On the other hand, not all types of raw materials are suitable and can be used to grow and multiply oyster mushrooms. One example is the use of oat seed as a medium. Research showed that it was not suitable for growing mushrooms because oat quickly softens and disintegrates when washed, soaked, and when given a pretreatment of prolonged submersion. In other studies, it was observed that at the beginning, mycelium could still grow well on the oat seed medium, but it would eventually die on the 21st day. Furthermore, other research also suggests the use of millet seeds as it produce a compact mycelium colony, pure white in color, and consistent growth. This is mainly because the millet seeds contain proteins, amino acids, fat, fiber, ash, essential minerals, and vitamins that are good for the growth of mycelium (Maziero and Zadrazil 1994; Sumiati and Djuariah 2005).

Mycelial Growth Weight

The results on the speed of mycelium weight gain are shown in Table 2. Based on the observations, kapok seed and corn seed medium (T3L1) (1.56 g d^{-1}) and kapok and tofu medium (T3L2) (1.93 g d^{-1}) had a relatively lower weight obtained as compared to control medium (T1L1) (2.07 g d^{-1}). Thus, the medium derived from the corn seeds gave the best result among the others regardless of the kind of medium starter used (Figures 2 and 3).

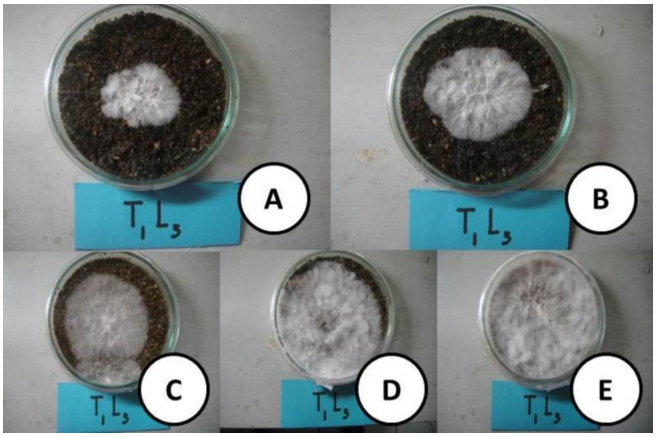


Figure 2. Horizontal mycelial growth (A) 1 cm; (B) 2 cm; (C) 3 cm; (D) 4 cm; (E) 5 cm

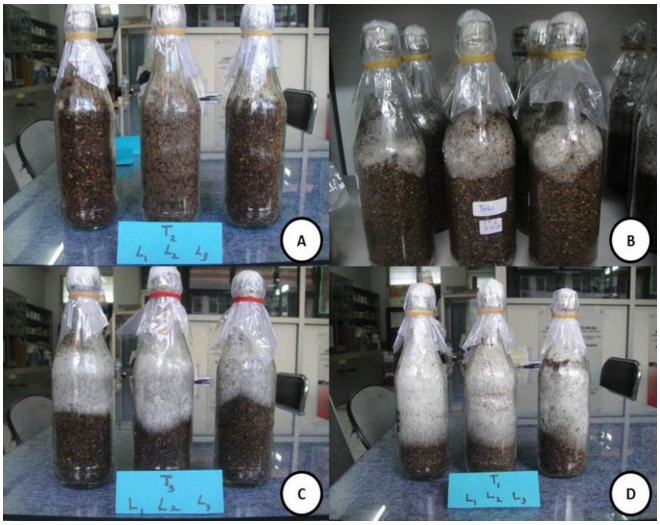


Figure 3. Vertical Mycelial Growth (A) Early vertical mycelial growth ; (B) Mycelia - 3 cm; (C) Mycelia - 7 cm; (D) Mycelia - 10 cm

Furthermore, oyster mushrooms were able to degrade lignin and make it as a source of energy for its growth. However, it cannot grow well by just relying on a one carbon source to degrade lignin. Thus, additional carbon source is needed to degrade lignin and other compounds. Moreover, the choice of agro-industrial waste to be used in the growing medium is also very important. A study on the use of arrowroot waste as a growing medium reported that the starch content of the arrowroot fiber cannot be decomposed by oyster mushroom, thereby making the nutrients unavailable for the mushrooms. However, oyster mushrooms would require other source of compounds in the form of cellulose, hemicellulose and lignin (carbohydrate compound bonding β 1,4 glycoside) as the main source of nutrition. As a medium, rice straw contains high hemicellulose and fair cellulose and lignin. The degree of polymer is much lower in rice straw, thus the medium could be easily and quickly decomposed by mushroom and the mycelium would grow well and fast (Heryogya et al. 2010).

Rice bran can be used as a medium starter to supply additional nutrients. Rice bran contains 10% structural

Table 2. Average mycelial growth weight of oyster mushrooms

Treatment	Weight and times of growth (g d^{-1})				Speed of weight growth of mycelium (g d^{-1})		
	Horizontal		Vertical		Horizontal	Vertical	Average
	weight	time	weight	time			
T1L1 (maize + rice bran)	23	14	75	30	1.64 ab	2.50 b	2.07 ab
T1L2 (Corn + Tofu Pulp)	23	14	85	32	1.64 ab	2.66 b	2.15 b
T1L3 (Corn + Soybean Seed Skin)	25	13	75	32	1.94 b	2.34 ab	2.13 b
T2L1 (*Leucaena + bran)*	25	20	77	45	1.25 ab	1.71 a	1.48 a
T2L2 (*Leucaena + Tofu Pulp)*	24	15	69	40	1.60 ab	1.73 a	1.66 ab
T2L3 *(Leucaena + Soybean Seed Skin)*	25	22	68	40	1.14 ab	1.70 a	1.42 a
T3L1 (Kapok + bran)	25	20	69	37	1.25 ab	1.86 ab	1.56 ab
T3L2 (Kapok + Tofu Pulp)	25	14	83	40	1.79 ab	2.08 ab	1.93 ab
T3L3 (Kapok + Soybean Seed Skin)	24	22	90	45	1.09 a	2.00 ab	1.55 ab
LSD	TN		14.53		0.843	0.77	0.66

*values with the same letter within a column are not significantly different at 5% LSD

carbohydrate, 7.5% total protein, 2.25% fat, and 7.5% variety of minerals (Nursiam 2012). Tofu waste, on the other hand, has enough protein to stimulate early mycelial growth. Tofu is a food commonly consumed by people in Indonesia. Tofu waste contains 414 kcal energy, 26.6 g protein, 41.3 g carbohydrates, 18.3 g fat, 19 mg Calcium (Ca), 29 mg Phosphorus (P), 4 mg Iron (Fe) and 0.2 mg Vitamin B1 (Herdiyana 2012).

Another factor that affects the growth of mycelium is the pH of the medium. The best mycelial growth observed in this research was at pH 8. Similarly, Wiardani (2010) explained that the level of acidity of the medium affects the growth of mushroom. A pH of 8 produces heavier weight compared to pH 7 and control. The average weight of mushrooms grown in a medium with a pH of 8 was 32.60 g. Moreover, according to Widyastuti and Tjokrokusumo (2008), the acidity of the medium is very influential on the growth of the oyster mushrooms. If the pH is too low or too high, this could lead to a stunted oyster growth. Thereby, the acidity of the medium should be regulated between pH 6-7 by using a chalk (calcium carbonate).

CONCLUSION

Leucaena and kapok seeds can be used as substitute for corn seed as a major medium for growing oyster mushrooms. Moreover, bran can be substituted by tofu -waste and soybean seed coat. Also, corn seeds are more compatible and effective when combined with the three agro-industrial wastes such as bran, tofu-waste, and soybean seed coat.

ACKNOWLEDGEMENT

Acknowledgement is given to DP2M, Higher Education, the Ministry of Research Technology and Higher Education, for providing the research grant.

LITERATURE CITED

Darlina E, Darliana I. 2008. Effect of dose bran in growing media on growth and yield of white oyster (*Pleurotus floridae*). Majalah Ilmiah Bulanan Kopertis Wilayah IV XX (6):32-38.

Fithrawan S. Engineering manufacture of oyster. 6 June 2011. <http://jamursekolah dolan.blogspot.co.id/2011/06.html >.

Herdiyana A. 5 September 2012. The benefit and content of tofu. <http://ekstrakjuskulit manggis.wordpress.com/tag/tabel-perbandingan-gizi-yang-ada-pada-tahu-dan-ampas-tahu/>.

Heryogya S, Nur H, Endah RL. 2010. Optimization production of grey oyster (*Pleurotus sajorcaju*) On soluble fiber mixture and rice straw. Jurnal Tek Pert.4(1):1-12.

Husen S, Santoso U, Wahyudi T. 2002. Effect of various sawdust on production and nutrition of three kinds of wood mushrooms. Jurnal Tropika 10(1):79-86.

Maziero R, Zadrazil F. 1994. Effect of different heat pre-treatments of wheat straw on its microbial activity and colonization by different tropical and sub-tropical edible mushrooms. World Journal of Microbiology & Biotechnology (10):374-380.

Nursiam I. 2009. The nutritional content of corn, soybean skin, bran, and cassava waste. INTANNURSIAM'S BLOG, <https:// intannursiam.wordpress.com/2009/12/01/ kandungan-nutrisi%C2%A0jagungbk-kedelaidedakonggok/>.

Pradita K. 2012. The concentration and frequency of giving biological fertilizer on growth and production of oyster.<http://

biologi.fst.unair.ac.id/wp-content/ uploads/2012/04/ Jurnal-Pradita-Kirana.pdf>.

Ramza S, Nurmiati, Periadnadi. 2013. Effect of acidity settings of sawdust media on growth and production of brown oyster (*Pleurotus cystidiosus* O.K. Miller.) Jurnal Biologi Universitas Andalas (J. Bio. UA.) 2(1):31-36.

Sumiati E, Djuariah D. 2005. Improvement of oyster production technology by time-varying immersion media growing powder wood saws. J. Hort 15(3):177-183.

Widyastuti N, Tjokrokusumo D. 2008. environmental aspects as critical success factors of oyster cultivation (*Pleurotus* sp.). Jurnal Teknologi Lingkungan 9(3):287-293.

Wuye RA. 2013. The addition of EM4 and composting time of growing media on growth and results of white oyster (*Pleurotus florida*). Agri-tek.14(1) : 1-23.

Philippine Journal of Crop Science (PJCS) Special Issue 2016 41(1):22-25

Diversity of Phylloplane Saprophytic Fungi on Monoculture Shallot (*Allium Cepa*) and its Potency as Microbial Antagonist to Purple Blotch Disease (*Alternaria Porri*) In East Java, Indonesia

Herry Nirwanto* **and Tri Mujoko**

University of Pembangunan Nasional "Veteran" Jawa Timur, Surabaya, East Java, Indonesia.
*Corresponding author, herry_n@upnjatim.ac.id

Purple blotch disease caused by *Alternaria porri* (Ellis) Cif. is one of the major diseases in shallots, with yield losses reaching as much as 40% (Wahyuno et al. 2003). Chemical and physical disease control measures, as well as the use of resistant varieties against purple blotch disease have not achieved satisfactory results in minimizing damage on shallot. This research explored the potency of various types of phylloplane and phyllosphere fungi as microbial antagonist to *A. porri* infecting shallot crops. Shallot samples were taken from several shallot-planting areas in Probolinggo, Malang, Kediri and Nganjuk District. The experiment was conducted from September 2009 to January 2010 at the Plant Pest and Disease Laboratory, Faculty of Agriculture, UPN "Veteran" East Java. Research survey was conducted in areas with an altitude of 150-600 meter above sea level (masl). Exploration was done to get the phylloplane and phyllosphere fungi from several varieties of shallot crops of different origins. The results showed that the most prevalent species found in all districts were *Penicillium* and *Stemphylium.* Analysis of phylloplane fungal diversity from Malang and Probolinggo isolates showed diversity index value of 2.99 and 3.54, respectively.

Keywords: *Alternaria porri*, antagonism, phyllosphere and phylloplane fungi, saprophytic, shallot

INTRODUCTION

Purple blotch disease caused by *Alternaria porri* (Ellis) Cif. is one of the major diseases in shallots, with yield losses reaching as much as 40% (Wahyuno et al. 2003). Chemical and physical disease control measures, as well as the use of resistant varieties against purple blotch disease have not achieved satisfactory results in minimizing damage on shallot. In addition, chemical fungicides pose hazards to the environment and human health. Chemical control could also lead to development of pathogen resistance and dwindled diversity of non-target organisms (Khamna et el. 2009). The use of biocontrol agents against pathogens is an established method of managing diseases with minimal negative environmental impact. Saprophytic fungi have shown potential antimicrobial properties against pathogenic fungi, and are therefore promising biocontrol agents. This research explored the community of various types of phylloplane and phyllosphere fungi on shallot crops and their potency as microbial antagonist to *A. porri.*

MATERIALS AND METHODS

Experiment Site

The experiment was conducted from September 2009 to January 2010 at the Plant Pest and Disease Laboratory, Faculty of Agriculture, UPN "Veteran" East Java. Research survey was conducted in areas with an altitude of 150-600 meter above sea level (masl). To get the phylloplane and phyllosphere fungi from several varieties of shallot crops of different origins, the samples of disease plants were selected from healthy plant in crop planting area.

Sampling. Shallot samples were taken from several shallot-planting areas in Probolinggo, Malang, Kediri and Nganjuk District. Samples were selected from healthy plants growing among diseased plants. Five samples per variety and location were selected and brought to the laboratory for isolation of saprophytic fungi.

Isolation of Saprophytic Fungi. Phylloplane and phyllosphere fungi were isolated based on the method by Mohamed and Abdel (2001). Healthy leaves which were cut into 1 cm fragments, washed with sterile water and dried by clamping between sterile tissue paper, were planted on potato dextrose agar (PDA) media with antibiotics to prevent bacterial growth. Fungal colonies that developed were counted, isolated and purified.

Isolation of Pathogen. *A. porri* was isolated from the leaves of shallot plants with purple spots. Leaves with spot were cut, washed in 70% alcohol followed by sterile water before planting on PDA media. The growing colonies of *A. porri* were isolated and purified.

Analysis of Diversity

Species diversity of isolated colonies of saprophytic fungi was analyzed using diversity indices calculated

using Simpson's Diversity Index equation (Oka 1993):

$$D = N(N-1)/\Sigma n(n-1)$$

where:
D = index of diversity
N= total number of individual
n = the individuals of each species

Antagonism Test
Phylloplane fungi of shallot obtained from purified cultures using 0.5-cm diameter cork bore were tested for antagonism against *A. porri* by inoculating on PDA medium .

Observation of the culture was made daily at 2-7 d after inoculation. The diameter of the *A. porri* colonies was measured and used to calculate inhibition of colony growth caused by the fungal antagonists. Percent inhibition was computed using the formula:

$$P = (R1-R2)/R1 \times 100\%$$

where:
P = % inhibition of *A. porri*
R1 = radius of the *A. porri* colony without phylloplane fungi
R2 = radius of the A. porri colony grown with phylloplane fungi

Identification of Saprophytic Fungi
Phylloplane fungus with the highest antagonistic effect on *A. porri* was selected and identified using conventional dichotomous key determination based on morphological characteristics of both mycelia and spores, colony color, colony growth rate, and other properties (Alexopoulos and Mims 1979).

RESULTS AND DISCUSSION

Identification and diversity of phylloplane fungi
There were seven major phylloplane fungal species identified from the different shallot varieties sampled from various districts in East Java (Table 1). The most prevalent species found in all districts were Penicillium and Stemphylium. Analysis of phylloplane fungal diversity from Malang and Probolinggo isolates showed diversity index value of 2.99 and 3.54, respectively (Figure 1). While from Nganjuk, the diversity index was not reported since it was very low. Also, from Kediri, the colony diversity could not be differentiated well because of domination by Trichoderma colony. The low diversity of phylloplane fungi in Nganjuk and Kediri was probably because this area had more rainfall than Probolinggo and Malang. The average rainfall and rainy days in Nganjuk, Kediri, Malang and Probolinggo are 105 mm, 6 HH; 121 mm, 7 HH; 30 mm, 8 HH; and 22 mm, 5 HH, respectively. The distribution of rainfall and rainy days in Nganjuk and Kediri showed that the study area is classified as wet climate with relatively high rainfall. In areas where rainfall is higher, farmers apply more chemicals.

Table 1. Saprobic fungal species isolated from different shallot varieties from various districts in East Java

Isolate		Saprobe
District	Variety	
Probolinggo	Lancur biru	*Gliocladium* sp.
		Stemphylium sp.
		Penicillium sp.
Malang	Philip	*Penicillium* sp.
		Fusarium sp.
	Thailand	*Penicillium* sp.
Nganjuk	Philip	*Fusarium* sp.
	Majalengka	*Stemphylium* sp.
		Trichoderma sp.
	Bauji	*Penicillium* sp.
		Stemphylium sp.
Kediri		*Curvularia* sp.
		Stemphylium sp.
	Thailand	*Curvularia* sp.
		Fusarium sp.
		Trichoderma sp.

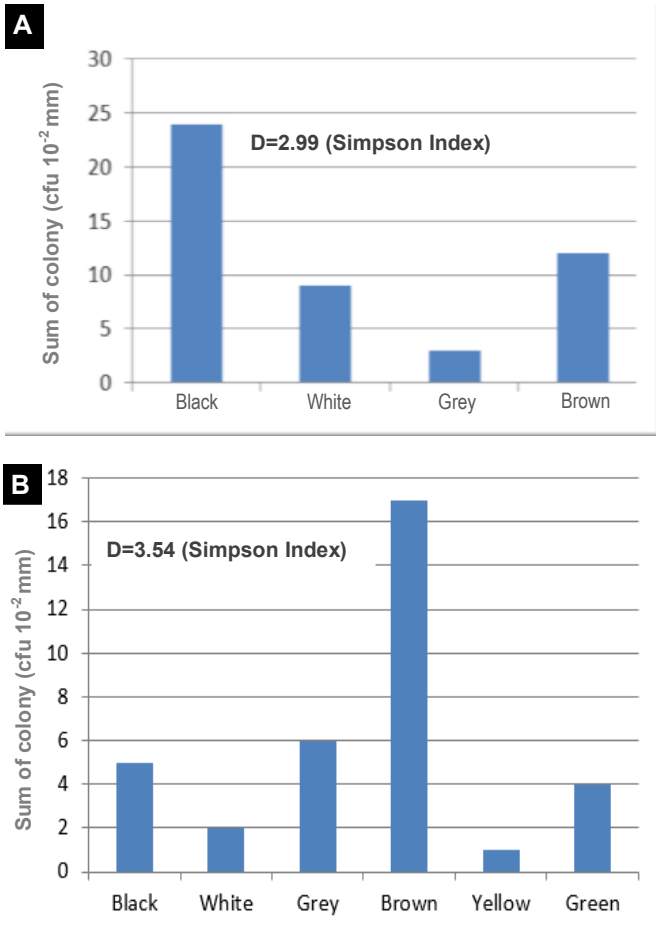


Figure 1. Colony color diversity of phylloplane fungi from Malang (A) and Probolinggo (B) shallot samples

Figure 2. Culture with different colony colors in petri dish and reaction tube

Figure 3. Diversity of colonies of Phylloplane fungi

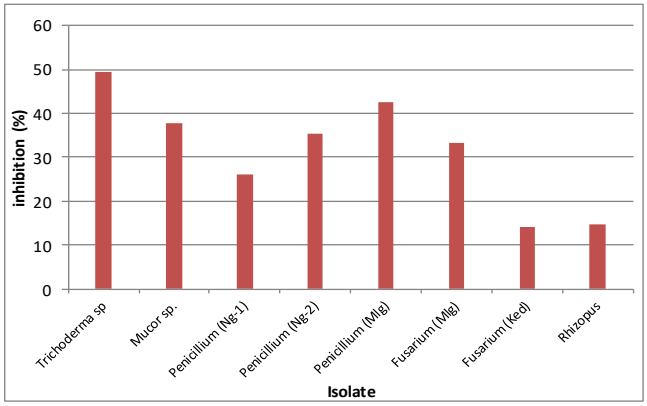


Figure 4. Inhibition potential of phyllosphere fungi against *Alternaria porri* after day 7

The index values suggest that fungal species diversity on the leaf surface of the samples is low, which could be due to the selective effect of fungicide treatment in shallot crops. The use of fungicides can reduce the number of species of phylloplane saprobic fungi

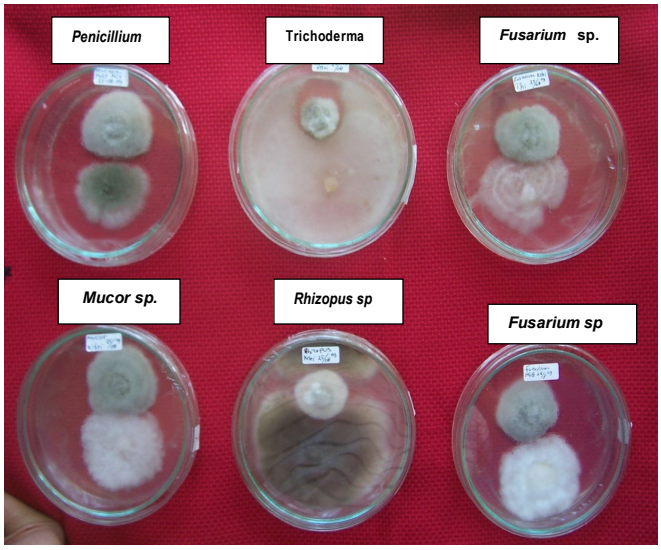


Figure 5. Diversity of colonies of Phylloplane fungi (lower side) against *A. porri* colony (Upper side) on PDA media

present on shallot crops (Blakeman and Williamson 1994). If the diversity becomes low, one or two species can become dominant (Oka 1995; Price 1997), and this decreased diversity could lead to instability of the community system, as high diversity promotes environmental stability (Price 1997; Pielou 1975). The dominance of *Penicillium* and *Stemphylium* also suggests the instability of shallot-growing environment.

Colonies of all phylloplane fungal isolates were velvety to downy or powdery in texture. They showed various shades of black, white, gray, brown, green, yellow, most commonly blue-green to grey-green with narrow border of black, yellowish, white, and brown (Figure 3).

Antagonism tests showed that phylloplane fungi inhibited pathogen growth (Figure 4). *Trichoderma* sp. isolate from Kediri and *Penicillium* sp. isolate from Nganjuk inhibited the growth of *A. porri* by more than 30% in the first observation. Other species inhibited

more than 30% *A. porri* growth in the succeeding observations. Figure 5 indicated the inhibition of Trichoderma through antibiosis mechanism as seen on the free zone between the two colonies.

CONCLUSION

The observation of antagonism tests showed that *Trichoderma* sp. and *Penicillium* sp. inhibited fungal colonies of pathogenic fungi by antibiosis, while the other fungi inhibited the growth of *A. porri* by competition. *Trichoderma* sp. and *Penicillium* sp. have better antagonist potency against *A. porri* than the other phylloplane fungi.

The diversity of fungal species of phylloplane saprobe on shallot crops from the four districts is relatively low. Dominant species was *Penicillium* sp.. Based on the inhibition test, *Trichoderma* sp. and *Penicillium* sp. were the most potent antagonists against *A. porri*, via antibiosis mechanism.

LITERATURE CITED

Alexopoulos CJ, Mims CW. 1979. Intruductory mycology. 3rd ed. John Wiley and Sons. New York. 613 pp.

Anonimous 2009. http:www.countrysideinfo.co.uk/ simpsons.htm

Blakeman, Williamson, 1994. Ecology of plant pathogens. CAB International. Biddles Ltd. Guildford. UK. 384 pp.

Fenn ME, Paul HD, Daniel MD. 1989. Effects of ozone and sulfur dioxide on phyllosphere fungi from three tree species. Appl Environ Microbiol. Feb 1989; 55(2): 412–418.

Gveroska B, Jugoslav Z. 2012. Trichoderma harzianum as a biocontrol agent against Alternaria alternata on tobacco. Applied Technoloies & innovations. Vol. 7. Issue 2: 67 -76

Imiaj A, Tae-Soo L. 2008. Antagonistic effect of trichoderma species on the *Alternaria porri* pathogen of onion blotch. World journal of agricultural Siences 4 (1): 13-17.

Kacwchai S, Soytong K, Hyde KD. 2009. Myco fungicides and fungal biofertilizers. Fungal diversity 38: 25-50.

Kyeong Su J, Hong Mo K, Bong Koo C. 2001. Purification and antifungal activities of an antibiotic Produced by *Gliocladium.* The Plant Pathology Journal 17(1):52-56

Mohamed A, Abdel-Sater. 2001. Antagonistic Interactions between fungal pathogen and leaf surface fungi of onion (*Allium cepa* L.). Pakistan journal of biological sciences 4 (7): 838-842, 2001.

Pielou EC. 1975. Ecological diversity. John Wiley & Sons, Inc., New York. 165 pp.

Price PW. 1997. Insect ecology. 3rd edition John Wiley and Sons Inc, New York, USA. 801 pp

.

Khamna S, Akira Y, Peberdy JF, Lumyong S, 2009. Antifungal activity of *Streptomyces* spp. isolated from rhizosphere of Thai medicinal plants. International journal of integrative biology. 6(3): 143.

Swer H, Dkhar MS, Kayang H. 2011. Fungal population and diversity in organically amended agricultural soils of Meghalaya, India Journal of Organic Systems, 6(2):2011. http://www.organic- systems.org/journal/ Vol_6(2)/pdf/JOS_6(2)_2011_03-12_Swer.pdf

Philippine Journal of Crop Science (PJCS) Special Issue 2016 41(1):26-29

Natural Medium for Growing Endophytic Bacteria from *Solanaceae*

Arika Purnawati*, Wiwik Sri Harijani and Wiwin Windriyanti

University of Pembangunan Nasional "Veteran" Jawa Timur, Surabaya, East Java, Indonesia.
*Corresponding author, arika_p@upnjatim.ac.id

Endophytic bacteria are important microorganisms that are potential biocontrol agents for many pathogens. Its growth requires the use of semisynthetic or synthetic medium, the ingredients of which make it difficult for farmers to use in the field. It is also expensive for propagation as biocontrol agents. The study focused on identifying natural ingredients as medium for growing the endophytic bacteria. The medium developed have natural ingredients like soybean, chicken broth, egg, worms, snail, and sorghum, which are easy to obtain by farmers. The factors were arranged in completely randomized factorial design for four isolates of endophytic bacteria from solanaceae in Malang, Indonesia, and five natural ingredients for the medium-corn flour, soybean flour, sorghum flour, snail flour, and worm flour. Result of the study showed that the bacteria grew in all of the media used. There is no difference in the incubation period, color, shape, and surface colony. However, the population in the snail flour medium at 10^7 cfu mL^{-1} is the highest ($7.5x10^6$ cfu mL^{-1}), thus, the best medium for growing endophytic bacteria.

Keywords: endophytic bacteria, biocontrol agents, natural medium, propagation

INTRODUCTION

Endophytic bacteria have been found in every plant, where they colonize the internal tissues of their host plant and can form a range of different relationships including symbiotic, mutualistic and commensalistic. Endophytic bacteria can also be beneficial to their host by producing natural products that have potential for agricultural use, having been isolated from flowers, fruits, leaves, stems, roots and seeds of various plant species (Christy and Sudha 2014; Kobayashi and Palumbo 2000).

Growth of endophytic bacteria are influenced by the medium and other growth factors. Growth factors are required by cells in small amounts because they fulfill specific roles in biosynthesis. The need for a growth factor results from either a blocked or missing metabolic pathway in the cells and can be added to culture media that are used to grow bacteria. Use of culture in liquid or solid medium depend on the purpose. It may be classified into several categories depending on their composition or use. Chemical (synthetic) medium is one in which the exact chemical composition is known, while semisynthetic medium is one in which the exact chemical constitution of the medium is not known (Todar 2012). Growth of bacteria also influenced by diversity of nutritional types found among bacteria like an organic salt, carbon, an organic nitrogen, amino acid, and vitamins (Goyal 2007) but the medium is usually expensive.

Endophytic bacteria as biocontrol agents of pathogens in Malang, Indonesia need a growing medium with a composition that is easy to use and inexpensive for propagation by farmers in the field. Based on the problem, natural materials such as corn flour, soybean flour, sorghum flour, snail flour, worm flour were used as media. All of them contain growth factors such as organic and inorganic elements, vitamins and other elements (Ahmad 2002; Syatrawati 2008).

The objective of this experiment is to study different natural media for growing endophytic bacteria from solanaceae in Malang, Indonesia.

MATERIALS AND METHODS

The factors were arranged in completely randomized factorial design for four isolates of endophytic bacteria from solanaceae in Malang, Indonesia, and five natural ingredients for the medium-corn flour, soybean flour, sorghum flour, snail flour, and worm flour.

Isolates of endophytic bacteria

Endophytic bacteria used for the experiment is a personal collection of the lead author. The bacteria culture used for the experiment was purified on NA (Difco): 8 g L^{-1}, pH 7.0, within 24 hr.

Preparation of the natural medium

Corn flour. Corn was shelled and cleaned, then dried for 1-2 d at 50 C until 15-18% moisture content was reached. The corn was ground and sieved into flour using a size 50 mesh.

Soybean flour. Soybeans were cleaned with flowing water, then soaked for 4 h while squeezing to

clean the epidermis. The soybeans were washed again with water for 15 min and drained. The beans were dried (1-2 d) then roasted for 10-15 min, and ground to flour.

Sorghum flour. Cleaned sorghum seeds were dried to 20% moisture content, soaked in water for 8 h, and dried again to 16% moisture content.

Snail flour. Fresh snail meat were removed from the shell then sun-dried for 3 d or by using a dryer until it reached 14% moisture content. The dried meat was milled and ground into flour.

Worm flour. Worms were washed and boiled in water for 3 min, then drained. After draining, the worms were cut into 1 cm pieces and washed again. The pieces were oven dried at 500°C for 4 h and mashed into flour.

Achatina flour. Achatina were allowed to stand for 2 d and 2 nights. Salt was added and stirred for 15 min, then drained for 15 min. After draining, it was washed again and boiled for 20 min then drained and dried. The process was repeated before the Achatina were sliced and dried again before grinding into flour.

Incubation of the bacteria in the different media

For the experiment, 20 g L^{-1} were measured from each flour sample. The medium is divided into liquid (without added agar) and solid medium (with 20 g L^{-1} of agar added). The media were sterilized using autoclave at 121 C, 1.5 atm for 15 min and then 10 mL of each sample is poured into a Petri dish. Endophytic bacteria colonies were inoculated into the different media then incubated at 28 C for 48 h. The incubation period, color, shape, surface colony and population of bacteria on natural medium using dilution technique at concentration 10^7 cfu mL^{-1}, at 24, 48, 72, 96 h were observed.

The data were subjected to analysis of variance and differences in treatment means were determined using LSD at 5% level of significant.

RESULTS AND DISCUSSION

Incubation within 24 h showed that the bacteria colony in all natural medium is white and round with shiny surface (Figure 1). Figure 2 shows that population in each natural medium is indicator of growth while Figure 3 shows the general pattern of growth. It showed 4 growth phases namely: lag phase, log phase, stationary phase and death phase. In adaptation phase, bacteria requires nutrition for growing, in log phase the growing is accelerated, in stationary phase there is a balance between death bacteria and growth bacteria, and in death phase the number of dead bacteria is higher than the living.

Incubation period and bacterial colony are the same in the natural medium as well as in the control medium, because nutrition in the medium did not affect the incubation period and the bacterial colony. Population of endophytic bacteria is different across media due to the influence of the nutritional content in each medium. Population in liquid media is higher than in solid media, because the bacteria mixes with the liquid medium, affecting the acceleration of cell division, resulting in high population. Todar (2012) stated that growth of bacteria is affected by major elements (C, H, O, N, S. P, K, Mg, Fe, Ca, Mn) and trace elements (Zn, Co, Cu, Mo). Razzak (2009) states that bacteria will grow best on agar plates where air readily diffuse into the colony, or on liquid medium that are shaken. Todar (2012) states that there are other factors like temperature, pH, and water activity that affect growth of bacteria.

Based on Figure 2, population of endophytic bacteria in both forms of media (solid and liquid) at 10^7 cfu mL^{-1} is highest in medium with snail flour at $7.5x10^6$ cfu mL^{-1} for liquid medium and $1.1x10^5$ cfu mL^{-1} for solid medium followed by soybean flour, corn flour, sorghum flour, worm flour and achatina flour. Population (cfu mL^{-1}) in solid natural medium are $6.9x10^6$, $6.7x10^6$, $6.5x10^6$, $7.0x10^6$, and $7.4x10^6$; and $1.05x10^5$, $1.03x10^5$, $1.02x10^5$, $1.07x10^5$, $1.06x10^5$ in liquid medium. Population in natural medium is significantly higher than in control medium—Nutrient Agar (NA) is $6.0x10^6$ cfu mL^{-1}, Nutrient Broth (NB) is $1.0x10^5$ cfu mL^{-1}. In all natural media, bacterial population in liquid medium is higher than in solid medium. An increase in population was observed at the 24^{th} hour for all solid and liquid natural media, and another significant increase at the 48^{th} hour. However, there is no increase in population at 72^{nd} until the 96^{th} hour.

Figure 3 shows that bacterial growth follow the pattern of logarithmic curve and is divided into four phases-the lag phase, log phase, stationary phase and death phase. Bacteria begin to divide and perform metabolic activity in the lag phase, 24 h after incubation. During the log phase or exponential phase, 48 h after incubation, bacterial mass and volume is increased; growth is balanced and speed increases. In the stationary phase, after 72 h, population begins to decline. The number of dead cells increases until the number of live cells equal the number of cells that die. The number of living cells remains the same in this phase, as there is no growth. In the death phase, after 96 h, the number of dead cells exceeded the number of living cells. Despite this decline in living cells, the number did not reach zero. A specific minimum number of the remaining living cells last for a long time in the medium.

The research conducted by Chikere and Udochukwu (2014) mentioned the use of nutrient agar (NA) and plate count agar (PCA), which cause increased counts of endophytic bacteria and increased incubation time, respectively. Counts obtained in NA were higher than in PCA followed by the soil extract agar (SEA), which had the lowest count and a longer bacteria lag phase due to the low nutrient content in SEA. The PCA formed more distinct colonies than NA and SEA. During the incubation period, colonies appeared on

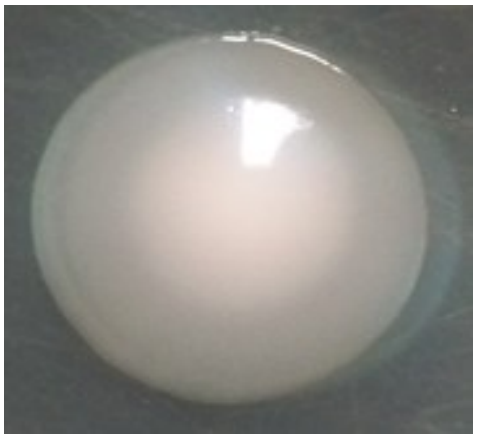


Figure 1. Microscopic colony of endophytic bacteria in natural medium (p.4.5x)

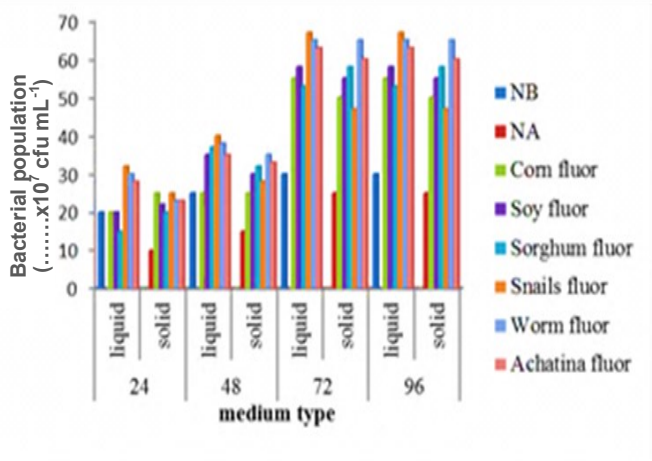


Figure 2. Histogram of population of endophytic bacteria

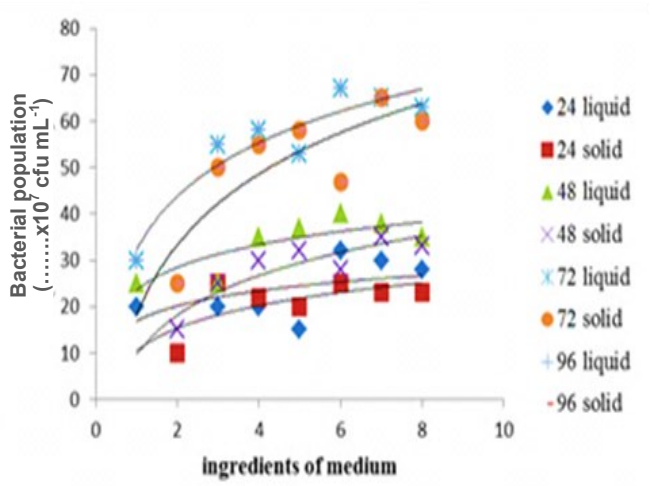


Figure 3. Curve of growing endophytic

the NA even on the 7th day. This characteristic of the NA to support a higher number of colonies for a long period of time could be attributed to its nutrient composition.

Typical growth curve of bacterial population can be divided into lag phase, exponential phase (log phase), stationary phase and death phase. In the lag phase, when a microbial population is inoculated into a fresh medium, growth usually does not begin immediately but only after a period of time, which may be brief or extended depending on the history of culture and growth conditions. Exponential phase is the phase of bacterial growth curve in which the bacterial cell numbers double in each unit time period. When the number of cells in an experiment is graphed on arithmetic coordinates as function of elapsed time, one obtains a curve with a constantly increasing slope. The rate of increase in cell number is initially slow but in later stage the cell numbers increases explosively. The rate of exponential growth varies between bacterial genera (i.e. Genetic characteristics of bacteria) and is also influenced by environmental conditions. Exponential growth does not occur in the stationary phase because the essential nutrients of the culture medium are used and waste products of organisms build in the environment. In the stationary phase there is no net increase or decrease in cell number. Cell functions such as energy metabolism and some biosynthetic processes go on. The death phase starts when bacterial population reaches the stationary phase, and the cells start dying. Cell death may be due to cell lysis. This is also an exponential process but much slower than that of exponential growth (microbeonline.com 2013).

CONCLUSION

From the experiment, in all solid and liquid media there is no difference in incubation period and colony. Population in agar and liquid medium with snail flour at 10^7 cfu mL^{-1} concentration is the highest.

ACKNOWLEDGMENT

The authors would like to thank DRPM Dikti for the fund support for this experiment.

LITERATURE CITED

Ahmad RZ, 2002. The local media for growth nematophagous fungi as a model. Proceedings of National Seminar on Animal Husbandry and Veterinary Technology, 444-449.

Chikere CB, U Udochukwu, 2014. Effect of growth media and incubation time on the culturability of soil bacteria. IOSR J. of Pharmacy and Biological Sci. 9:6-9.

Christy PH, Sudha SS, 2014. A study on the potential of endophytic bacteria as biocontrol agent. Scrutiny International Research J. Biological and Environment. 1(10): 7-11.

Goyal D. 2007. Methods of studying microorganisms. Depart of Biotechnology and Environmental

Sciences Thapar University. Patiala 305:105-110.

Kobayashi DY, Palumbo JD. 2000. Bacterial endophytes and their effects on plants and uses in agriculture, p. 199-233. *In* C.W. Bacon and JF. White (ed.). Microbial endophytes. Marcel Dekker, Inc. New York 310:199-233.

Microbeonline.com. 2013. *T*ypical growth curve of bacterial population.410:11-20. Date accessed August 2014.

Razzak L. 2009. Nutrition and growth of bacteria. www.uobabylon.edu.iq.1-5. Date accessed July 2014.

Syatrawati. 2008. Production of biofungicide compound witn *Gliocladium* sp. active several waste organic media. J. Agrisistem J. 4 (2):121-127.

Todar K. 2012. Nutrition and growth of bacteria. Online Texbook of Bacteriology. www.textbook of bacteriology.net. 25-35. Date accessed July 2014.

Philippine Journal of Crop Science (PJCS) Special Issue 2016 41(1):30-35

Application of Biodegraded and Modified Starch Waste Water on Biogas and Liquid Fertilizer Production

Edi Mulyadi[1*], Akhmad Fauzi[1], Soemargono[1] and Rudy Laksmono[2]

[1]University of Pembangunan Nasional "Veteran" Jawa Timur, Surabaya, East Java, Indonesia;
[2]University of Pertahanan Indonesia. [*]Corresponding author, ed1mulyad1@yahoo.com

PT Lautan Warna Sari (LWS) which produces modified starch-based raw materials at 200 tons per day can now use biogas as energy alternative. The high content of waste biomass has high economic value when processed into biogas. Proper waste handling only slightly remove the odor. Based on field observation, the formation of gas is characterized by the emergence of gas bubbles arising from the equalization basin. It raises the idea for managing and processing of waste into biogas. Biogas reactor (digester) models are made of plastic polyethylene balloon mounted on the anaerobic pond and placed half buried in the ground in order not to be separated when the product reaches the peak bio gas. Biomass intake holes and drain holes arranged to prevent blockage. The prototype digester used balloon model with a capacity of 17000 m^3, biogas purification unit is equipped with a capacity of 5.8 m^3 min^{-1}. The application of biogas as modified starch product dryers can replace the diesel fuel. The best production capacity of 4.6 m^3 min^{-1} in biodigester occurred at 35^oC temperature condition. The ratio of waste discharge to the flow of wash water utilizing the cassava as eddy currents was 1:0.5 and was capable of producing 42% methane gas. Moreover, purifying the biogas increased the methane gas content up to 73%.

Keywords: biogas, biomass, digester balloon, gas purifiers, modified starch

INTRODUCTION

PT (LWS) can produce modified cassava starch-based raw materials with a capacity of 200 tons per day or 40 tons of products. The high content of waste biomass may have an economic value when processed into biogas. However, the waste when left alone causes environmental pollution in the form of bad odor around the plant. Even proper waste handling could only slightly remove the odor. Based on field observation, gas is formed from the equalization basin. This leads to the idea for managing and processing the waste into biogas. In addition to the liquid waste, industrial solid waste is also produced which is being disposed off without any treatments. Both types of waste are raw materials for biogas, and the processing of them in digester produces not only biogas but also solid residue that can be used as fertilizer (Buren and Arnott 2004; Reese and Thompson 2008), and a plan was developed to combine the epidermis and solid impurities. The methane content of the biogas produced from wastewater treatment is low (<40%) and this limits its use as dryer for modified starch products. Biogas that comes out of digester still contains a high amount of impurities and therefore needs to be purified before it can be used directly to dry modified starch products.

Waste water is produced from different processes of production in the starch industry. These wastes are produced from cassava washing, extraction, separation, modified starch production, filtration, and pressing. The waste contains organic substances such as Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD). The elimination of the parameters allows the production of 80-90% organic pollutants and formation of methane gas from organic substances. The common types of biogas reactors are: 1) fixed dome reactor, 2) floating reactor, 3) balloon reactor, 4) horizontal reactor, 5) hole land reactor, and 6) ferro cement reactor. The balloon type reactor which uses a plastic material, is widely used at the household level since the cost of construction is cheaper and more efficient (Mulyadi 2010). The reactor consists of a section without a bulkhead and serves both as digester and gas storage. The organic material is located at the bottom because it has a greater weight than the gas and it will fill in the cavity above. University of UPN "Veteran" Jawa Timur did a lot to develop and innovate the application of this type of reactor but the design capacity was less than 20 t. The factors to consider in the development of bio-digesters include anaerobic environment and temperature (30-50$^{\circ}C$), that are important in the growth of mesophilic bacteria.

The parameters which affect the operating conditions of the bio-digester reactor (Dahlman 2012; Sadi 2010) are: (1) the degree of acidity (pH) which should be between 6.6-7.0 and should not be below 6.2; (2) the ratio derived from the biomass ranging 25 -30; (3) The nutrition which may include ammonia (NH3) as a source of nitrogen, and minerals of nickel (Ni), Copper (Cu), iron (Fe), phosphate (PO4), and zinc (Zn); and (4) feed concentration wherein the recommended biomass concentration should be from 0.26 to 0.3 kg L^{-1} .

According to Mulyadi (2012), adding a digester mixing function to obtain a homogeneous mixture of the substrate increases the frequency of collisions and turbulence so that the bio conversion will also increase. The biodegradation process takes place in two stages (Scott and Keddy 2006), anaerobic and aerobic processes.

This study aimed to produce a detailed design and prototype of a digester made up of 17,000 m^3 balloon equipped models with moving bed purification adapted to produce digester gas and to improve the gas purification process by designing a fixed, compact and adaptable gas digester which can utilize local ingredients.

MATERIALS AND METHODS

Raw Material

The raw materials used in this study were obtained from several parts of the PT LWS waste stream in Lampung. The base raw material was 200 t d^{-1}. The different waste materials collected in the form of solids or sludge were cassava mushy/wet at 500 kg d^{-1}, cassava press at 10 t d^{-1}, slurry-off grade wet at 200 kg d^{-1}, epidermisat 4 t d^{-1} and solids at 8 t d^{-1}. These biomass were the source of raw materials which were converted into alternative energy, biogas, bio-ethanol, and fertilizers in an anaerobic digester.

The calculations on the digester reactor was based on the data adjusted for the potential raw materials and the amount of waste biomass that were processed. The optimum composition for the reactor wake was 80% biomass and 20 % liquid.

Equipment

The reactor design model of the balloon had two parts: (1) the upper part which served as the site for the breakdown of materials and biogas storage (gasholder), and (2) the bottom part which served as the home for both acid-forming bacteria and methane-forming bacteria.

The digester used was built in the field and was powered by PT LWS Lampung. The upper portion of the biogas reactor was made of polyethylene while the bottom portion was made of excavated soil. The bio-digester reactor was equipped with a baffle which stirred the mixture as the biomass feed tubs and water enter the reactor. This allows a well mixing process that could run well and has rapid gas formation. The calculation of biogas produced in the digester was based on the theory similar to the prototype digester units.

The Biogas Fermentation Instrument with a volume capacity of 17,000 m^3 consisted of 3 parts. The 1st part was the framework units of permanent anaerobic pond which was 1-2 m deep. The 2nd part is the balloon digester unit which was prepared 3 times the size of the anaerobic pond and had an area of 17,000 m^2. The 3rd part was the skeletal remain unit, constructed from bricks that were arranged to resemble a tank with 17,000 m^3 capacity. The skeletal remain unit served as the foundation and support for the balloon digester unit (Figure 1).

The biogas produced in the digester was directed towards the purifying device and then stored in a gas holder before it was used as a fuel burner (Figure 2). The gas purifying equipment was designed and constructed in the engineering workshop using the appropriate technology of LPPM UPN "Veteran" East Java.

The gas holder or reservoir was made of plastic while the stabilizer tanks were made of Stainless Steel type 304 (SS 304). The gas purifying device was equipped with SS 304 plumbing pipe with various sizes which served as the primary network. The secondary network was made of galvanized pipe which was connected to the water heater. The burner for bio gas production run semi-automatically and the equipment were assembled automatically.

Products Usage

Modified starch industry waste (PT Tunas Jaya Lautan, Teluk Dalem Ilir, Kecamatan Rumbia Lampung Tengah) is used for biogas production in the form of liquid waste from the chemical vacuum filter processes, nonchemical vacuum filters, pressed cassava, separator and laundering. The waste was collected from the three settling ponds which were arranged in series. Digester reactors used tangible balloon. The biogas product was used solely by PT LWS as modified starch dryer energy. Biogas quality was improved through different treatments. One was through the removal of hydrogen sulfide, water, and carbon dioxide. Hydrogen sulfide gas is a toxic substance which causes corrosion. Hence, the maximum allowable concentration for hydrogen sulfide gas was set to 5 ppm. Another treatment was the removal of carbon dioxide content. And the last was the removal of water content in the biogas to lower the ignition point and prevent corrosion on biogas.

Meanwhile, the sludge digester products can be processed into solid and liquid organic fertilizer using an extractor and continuous granulator. These activities allow the production process to become efficient and have zero waste.

Digester Optimization

The requirements of the test equipment were determined. The instruments were calibrated using flow meter and manometer. The anaerobic fermentation of biomass were analyzed by determining the levels of the resulting gases such as methane, carbon dioxide and the gas time-out through gas chromatography (GC). The gases formed from the fermentation process were subjected to purification which was carried out by using gas absorber equipment. This equipment absorbed H_2S gas formed in water. The process was based on the solubility of H_2S gas at 320 cc gas 100 cc H_2O^{-1}. The CO_2 reacted directly with NaOH solution while the CH_4 gas did not react with NaOH. The reaction is presented in the


Figure 1. The left (a) and right (b) side of the skeletal remain unit of the digester

following equations:

$$CO_{2(g)} + NaOH_{(aq)} = NaHCO_{3\,(aq)} \quad (a)$$

$$NaOH_{(aq)} + NaHCO_{3\,(aq)} = Na_2CO_{3(s)} + H_2O_{(l)} \quad (b)$$

From equations (a) and (b), the derived equation is:

$$CO_{2(g)} + 2\,NaOH_{(aq)} = Na_2CO_{3(s)} + H_2O_{(l)} \quad (c)$$

Based on equation (c), the reaction of CO_2 with NaOH leads to a lower ratio of CO_2 to CH_4 concentration. Each adsorber column contained zeolites and silica solids which served as absorbent to the residual gases that which were not absorbed in the previous tool. The energy contained in biogas depended on the concentration of methane gas. The higher the content of methane gas, the greater the temperature required to heat the biogas.

The average biogas production was 4.8 m^3. The quality of biogas was obtained from 3 sampling times. The methane, water, and carbon dioxide content of biogas were determined before and after the purification process.

RESULTS AND DISCUSSION

Biogas formation was slow during the initial production process, hence, there is a need to accelerate the growth and formation reactions. It is necessary to effectively stir the reactant materials to increase the collision of materials. Then the digester liquid from the water used in the process could flow to wash cassava. The quality of waste water obtained from these processes was shown in Table 1. In PT LWS's site, there were 4 sources of waste water, each with its own characteristics. All the waste water then flowed together into the digester to be processed into biogas.

The physical and chemical properties of the waste water from the water digester when released into the aerobic pond is shown in Table 2. These values indicate good performance of the anaerobic digester in decomposing the organic pollutants. It is important because the waste water cannot be released into the receiving water body unless it meets the quality

Figure 2. The biogas purifying device

standards of Total Suspended Solid (TSS). These conditions correspond to the conditions on the ground at the aerobic pond.

Table 1. Physical and chemical properties of the waste water from PT LWS equipment

No.	Source	Parameter			Max allowable limit			Method		
		pH	COD (mg L^{-1})	TSS (mg L^{-1})	pH	COD (mg L^{-1})	TSS (mg L^{-1})	pH	COD (mg L^{-1})	TSS (mg L^{-1})
1	Starch Separator	4.47	6899.2	950	6 - 9	300	100	SNI 06-6889.11 - 2004	SNI 06-6889.15 - 2004	SNI 06-6889.3 - 2004
2	Sediment tanks for starch	3.41	6522.9	110	6 - 9	300	100	SNI 06-6889.11 - 2004	SNI 06-6889.15 - 2004	SNI 06-6889.3 - 2004
3	Tapioca purifier	6.48	533.1	1120	6 - 9	300	100	SNI 06-6889.11 - 2004	SNI 06-6889.15 - 2004	SNI 06-6889.3 - 2004
4	Accumulator of waste of modified starch	4.83	1285.8	830	6 - 9	300	100	SNI 06-6889.11 - 2004	SNI 06-6889.15 - 2004	SNI 06-6889.3 - 2004

Table 2. Physical and chemical properties of the released waste water from water digester

No.	Source	Parameter			Max allowable limit			Method		
		pH	COD (mg L^{-1})	TSS (mg L^{-1})	pH	COD (mg L^{-1})	TSS (mg L^{-1})	pH	COD (mg L^{-1})	TSS (mg L^{-1})
1	From digester to aerobic pond	7.79	219.5	300	6 - 9	300	100	SNI 06-6889.11 - 2004	SNI 06-6889.15 - 2004	SNI 06-6889.3 - 2004
2	From aerobic pond, discharged into the river	7.81	62.72	130	6 - 9	300	100	SNI 06-6889.11 - 2004	SNI 06-6889.15 - 2004	SNI 06-6889.3 - 2004

These conditions indicate proper water quality to be discharged into receiving water bodies, and is even good enough to be recycled and reused. Thus it shows the quality of environmental management leading to zero waste. The process of formation of methane gas (CH_4), which occurs in an anaerobic digester, shows perfection in the process of biodegradation of organic pollutants by utilizing anaerobic and aerobic bacteria and bacterial acidogenic methanogenic. Furthermore, the methane gas in the purification process was used as fuel for drying starch modification.

The average methane, carbon dioxide, and water content of the biogas before and after the purifications is presented in Table 3. The table shows significant methane content increase after the purification, which means the biogas is much more ready to be used as fuel.

The biogas formed and the percentage of component gases as influenced by the varying ratio of waste water flow are shown in Table 4. The rate of raw material production was 80 ton d^{-1} when the study was initiated. The gas pressure in the reactor digester, biogas production and gas content (%) were influenced by the ratio of the addition of wash water flow. The gas product of the raw materials increased with increasing fermentation time. The levels of methane gas (CH_4) increased with the increasing duration of the fermentation process, until it reached the maximum value on d 12 and a decreasing trend can be observed afterwards. Biogas is utilized by PT

Figure 3. Digester gas holder balloon

Figure 4. Aerobic pond after anaerobic process in the digester

LWS only as energy dryers and modified starch products with an average production rate of 4 m^3 min^{-1}. Considering that this value is equivalent to the energy need of modified starch factory of PT LWS which is 60 L h^{-1} of diesel fuel or 350 kg hr^{-1} firewood, then biogas can be an alternative energy source to diesel or firewood which are commonly used to provide energy to dry flour. Biogas has an advantage over diesel and firewood because it generates clean, blue flame and is able to stabilize the temperature of the drying process, which in the end increases the efficiency of the process by more than 27%. Methane, the main component of biogas has enough heating value of about 6200 kcal m^{-3}. Pure methane gas has a caloric value of 8900 Kcal m^{-3}. The initial production of methane gas contained in the biogas occurred when the tapioca industrial waste was digested for 5 d and the peak of methane production occurred at 16 d. The by-product of biogas could be used as solid and liquid organic fertilizers.

Table 3. Composition of biogas before and after purification

Component	Digester product concentration (%)	Percent concentration after purification
Methane	42	73
Carbon dioxide	41	6.0
Water	8	0.04

Table 4. Biogas and the amount of component gases produced in response to the varying proportion of discharged waste water from the digester

Proportion of the waste wash water flow Raw (Part)	Time (d)	Gauge Pressure (mm H_2O)	Biogas product (m^3 min^{-1})	Proportion in biogas (%)		
				CH_4	CO_2	water
1:0	1	4	0.75	12	40	10
	4	22	1.1	26	32	12
	8	66	1.8	34	29	7
	12	72	2.4	40	23	12
	16	80	2.6	40	29	9
1:0.25	1	4	0.8	16	36	11
	4	34	1.2	30	22	6
	8	88	2.4	36	23	9
	12	89	3.8	45	19	7
	16	88	2.9	42	26	12
1:0.5	1	6	0.92	20	40	12
	4	75	1.5	34	30	9
	8	90	2.8	40	33	11
	12	95	4.6	48	40	10
	16	90	3.6	44	42	9
1:0.75	1	10	0.95	22	42	11
	4	77	1.5	38	36	12
	8	97	3.4	39	40	11
	12	102	4.4	44	49	13
	16	93	3.8	44	47	9
1:1	1	10	1	24	52	8
	4	80	1.4	39	50	11
	8	95	3.6	41	54	10
	12	100	4	47	59	7
	16	90	3.8	40	60	8

Table 4 shows the varying amount of methane and carbon dioxide in the produced biogas in relation to the proportion of the raw materials. In addition to the waste from four sources as explained before, leftover water from an earlier process of cassava washing could also be mixed with the waste to increase the amount of produced biogas. The proportion between the waste and the leftover water is shown in the first column, and it can be seen that the amount of the waste remains constant at 1.

CONCLUSION

This study on the biodegradation of modified starch industry waste water with anaerobic process in digester has drawn the following conclusion:
1. The digester can be designed to produce 17,000 m^3 of methane from tapioca waste when the process is accompanied by eddy circulation.
2. The formation of biogas in the digester can completely eliminate odor pollution around the plant and can degrade the toxic COD more than 100 times.
3. Once the biogas is purified and becomes odorless, it can be used as an alternative fuel for diesel and firewood in tapioca industry.

ACKNOWLEDGEMENT

The authors are grateful to the DP2M DIKTI (RAPID scheme) that provided financial support to conduct the study. The authors extend their gratitude to the PT LWS where all the research activities were conducted.

LITERATURE CITED

Buren A, Arnott M. 2004. Biogas/Biofertilizer Business Handbook Manual: Peace Corps. Chinesse Biogas Manual. Intermediate Technology Publication Ltd. 186p.

Dahlman J. 2012. Technologier demonstrated at echo: floating drum biogas digester. http:// www.echonet.org.

Mulyadi E. 2012. Study piston fixed dome digester for optimization of biogas production process (Kajian digester kubah tetap berpiston untuk optimasi proses produksi biogas). In: Utilization of research results to support the empowerment of the local economy and industry. Proc. National Seminar. Surabaya.72p.

Sadi MA. 2010. Design and building of biogas digester for organic materials gained from solid waste:

First Edition. Faculty of Graduate Studies. An -Najah National University Press. Nablus-Palestine. 131 p.

Scott M, Keddy SM. 2006 Anaerobic digestion of the organic fraction of municipal solid waste. University of Florida. Department of Environmental Engineering Sciences. 193p.

Thompson RS. 2008. Hydrogen production by anaerobic fermentation using agricultural and food processing wastes Utilizing a two- stage digestion system. (MS Thesis). Utah State University. 76p. (Available at Utah State University Merrill-Cazier Library or https://digitalcommons.usu.edu/etd/208).

Philippine Journal of Crop Science (PJCS) Special Issue 2016 41(1):36-40

Vegetative Compatibility Group in Pathogenic Isolates of *Fusarium oxysporum* f.sp. *cepae* Causing Twisting Disease in Shallot

Sri Wiyatiningsih[1*], Arif Wibowo[2] and Endang Triwahyu P.[1]

[1]University of Pembangunan Nasional "Veteran" Jawa Timur, Surabaya, East Java, Indonesia; [2]Gadjah Mada University, Yogyakarta, Indonesia. *Corresponding author, sri.wiyatiningsih@upnjatim.ac.id

Twisting disease caused by *Fusarium oxysporum* f.sp. *cepae*, showed the highest disease intensity average of 77.90% in shallot grown in the paddy fields of Nganjuk manifold Vertisol soil, without rotation with rice, during rainy season. The symptoms of twisting disease on shallot caused by *Fusarium oxysporum* f.sp. *cepae* in some shallot production centers vary widely. This raises the suspicion that there are differences in virulence caused by VCG differences in pathogen isolates. Thus, the aim of this study was to obtain isolates of *Fusarium oxysporum* f.sp. *cepae* from plant materials collected from several shallot production centers and determine if the isolates obtained are from the same VCG group or not. To obtain the isolates of *Fusarium oxysporum* f.sp. *cepae* from diseased plant tissue, selective media is used in the isolation method. The Vegetative Compatibility Group (VCG) test of *Fusarium oxysporum* f.sp. *cepae* isolates was conducted by pairing nitrate nonutilizing (nit) mutants that are generated on media containing 1.5-4.0% potasium chlorate. There were 8 isolates of *Fusarium oxysporum* f.sp. *cepae* taken from the symptomatic cultivar varieties in the central areas of shallot production. It was found that among the 8 isolates of *Fusarium oxysporum* f.sp. *cepae*, only 4 of them have different VCG. These four were characterized as the four of different Ras, isolates A, B, C and D and Ras 1, 2, 3 and 4.

Keywords: *Fusarium oxysporum* f.sp. *cepae*, shallot, twisting disease, vegetative compatibility group

INTRODUCTION

Fusarium oxysporum is a common soil borne plant pathogen with a worldwide distribution. Strains of *Fusarium oxysporum* have been divided into formae speciales on the basis of virulence on a particular host or group of hosts. Further subdivisions of formae speciales into races often are made based on virulence to a particular set of differential host cultivars that vary in disease resistance (Corell 1991). Within species, however, there is a high level of host specificity with over 120 described formae speciales and races capable of causing vascular wilt diseases of many agricultural crops (Amstrong 1981).

Twisting disease caused by *Fusarium oxysporum* f.sp. *cepae*, showed the highest disease intensity average of 77.90% in shallot when grown in the paddy fields of Nganjuk manifold Vertisol soil, without rotation with rice, during rainy season. The symptom of twisting disease is pseudo stem and leaves that grow longer and curved. The color of leaves is pale green, but it does not wither. The bulb of sick plants are smaller, and do not show any decay in the tuber and root. On further conditions, the plants become dry and die. In the field, twisting disease symptoms begin to appear on the plant approximately within 20 d from planting. Experiments in greenhouse showed that twisting disease has an incubation period of 14 d. Koch's postulates proved that *Fusarium oxysporum* f.sp. *cepae* causes twisting disease (Wiyatiningsih 2012).

Strains of *Fusarium oxysporum* of various formae speciales have been grouped on the basis of vegetative compatibility. This approach provides a means for characterizing subspecific groups based on genetic composition of the fungus rather than the host pathogen interaction. In addition, vegetative compatibility allows the characterization of the non-pathogenic portion of population (Puhala 1985). Strains of *Fusarium oxysporum* can readily be tested for vegetative compatibility by pairing nitrate non utilizing (nit) mutants that are generated on media containing 1.5-4.0% potassium chlorate (Correll et al. 1987; Puhala 1985).

Colony growth on this medium is usually greatly restricted. After 7-14 days, fast growing, chlorate-resistant sectors can usually be observed originating from the initially restricted colony. When these chlorate-resistant sectors are grown on a minimal medium containing nitrate nitrogen as the only nitrogen source, they have a thin typically of expansive morphology with no aerial mycelium indication of a nit mutant. Then nit mutants can be phenotypically classified by their growth on a basal medium amended with one of several different nitrogen sources. Phenotypical district mutant (particularly) nit 1 and nit mutant, when paired on minimal medium containing nitrate as the sole nitrogen sources, will produce a zone of wild type growth (aerial mycelium) where the two nit mutant colonies come in contact. This occurs as a result of

hyphae fusion and nutritional complementation in the heterokaryotic cells. This complemementation nit mutant test can be used to test other strains for vegetative compatibility. A heterokaryon can form only between nit mutants of vegetative compatibility strains. Thus, isolates that show vegetative compatibility belong to the same VCG (Correll 1991).

The symptoms of twisting disease on shallot caused by *Fusarium oxysporum* f.sp *cepae* in some shallot production centers vary widely. This raises the suspicion that there are differences in virulence caused by VCG differences in pathogen isolates. Thus, the aim of this study was to obtain isolates of *Fusarium oxysporum* f.sp. *cepae* from plant materials collected from several shallot production centers and determine if the isolates obtained are from the same VCG group or not.

MATERIALS AND METHOD

Isolation of *Fusarium oxysporum* f.sp. *cepae*
Plant tissues infected with moler disease of shallot were collected from three different planting areas i.e Bantul, Nganjuk, and Brebes regency. Diseased plants were cleaned and disinfected using 70% ethanol, chopped into 0.5 cm long and placed on sterile petri dish lined with 3 cm diameter sterile filter paper. The culture media Commad was poured into sterile petri dishes to solidify before placing the diseased plant sample on the media and allowed to incubate at room temperature (28-30°C) for 3 d. After 3 d, the growth of fungus was observed though the colony formed. The media Commad was used because it is a selective media for *Fusarium oxysporum* (Nelson et al. 1981; Wakman 2004; Leslie and Summerell 2006). A single spore from the growing fungus was taken out of the PDA medium and isolated. This was done several times until the original breed of fungus was obtained, and then was identified both macroscopically and microscopically. Identification of the isolated fungus was further done by observing the colony and microscopic morphology in growth medium of Carnation Leaf-piece Agar (CLA) (Leslie and Summerell 2007; Salleh B 2007 *personal communication*). The fungus that grew on some pieces of carnation leaf in CLA media was observed microscopically. Sporrodochium as a macroconidium maker was observed to form on each piece of carnation leaf. Some semi-permanent preparation was also made by taking some fungus colony and mounting it on clean glass that contain drops of lactophenol cotton blue dye. The colony in the dye drops was then covered with glass flap and pasted with nail polish. The fungus on the preparate was observed and its morphology was drawn using Olympus microscope CX31, micrometer 0.01 mm, and image prism. The results from this observation was then used as the identification base of fungus morphology that would be isolated from the soil and calculation of its population density by referring to the morphological nature (characteristics) of fungus in accordance with its bibliography.

Testing the Vegetative Compatibility Group (VCG) of *F. oxysporum* f.sp cepae isolates.
This test was conducted to determine if the isolates of *F. oxysporum* f.sp *cepae* obtained are from the same group or not. Nit mutant of *F. Oxysporum* f.sp *cepae* was obtained by inoculating the pure culture disc of fungus in the minimal agar medium added by chlorate (MCM). Culture was incubated at room temperature for 7 d. Observation was done every day to know whether the growth of fungus isolate was faster than the colony of *F. oxysporum* f.sp *cepae* or if growth was hampered. Then, all the fungus isolates were transferred to the minimal agar medium (MM) with fusarium. The isolate colony that grew thin and fast and did not form air mycelium was nit mutant of *F. oxysporum* f.sp *cepae* based on Corell et al. (2002).

The complementation test was conducted by inoculating the breeding disk with the two nit mutants obtained from the different parent with the range of 1-3 cm at the medium MM incubated at room temperature for 7 days. Furthermore, the vegetative compatible nit mutant could be complement one another by making heterocarion at the medium MM that could be observed from the molding of thick and compact mycelium where the colony from the two nit mutant touch (Corell et al. 2002).

RESULTS AND DISCUSSION

Isolates of *Fusarium oxysporum* f.sp *cepae*
Figure 1 shows the isolate of *Fusarium oxysporum* f.sp. *cepae* taken from shallot plants with twisting disease symptomatic from cultivars and central areas of shallot production as follows:

A. Blue Cultivar was taken from the farmland of Parangtritis, Bantul
B. Blue Cultivar was taken from the farmland of Parangtritis, Bantul
C. Blue Cultivar was taken from the farmland of Sanden, Bantul
D. Yellow Cultivar was taken from the farmland of Kemurang Kulon, Brebes
E. Bima Cultivar was taken from the farmland of Kemurang Kulon, Brebes
F. Bima Cultivar was taken from the farmland of Kemurang Kulon, Brebes
G. Phillip Cultivar was taken from the farmland of Ngadiboyo, Nganjuk
H. Bauji Cultivar was taken from the farmland of Ngadiboyo, Nganjuk

Identification was done to make sure that the fungus, as a result of isolation, was the pathogen of twisting disease based on previous research which reported that *Fussarium oxysporum* f.sp *cepae* was the pathogen of twisting disease. *Fussarium oxysporum* f.sp. cepae cultured in Potato Dextrose Agar showed faster growth of colony with diameter larger than 2-5 cm are after 4 d, with its breeding color white up to violet (pale purple).

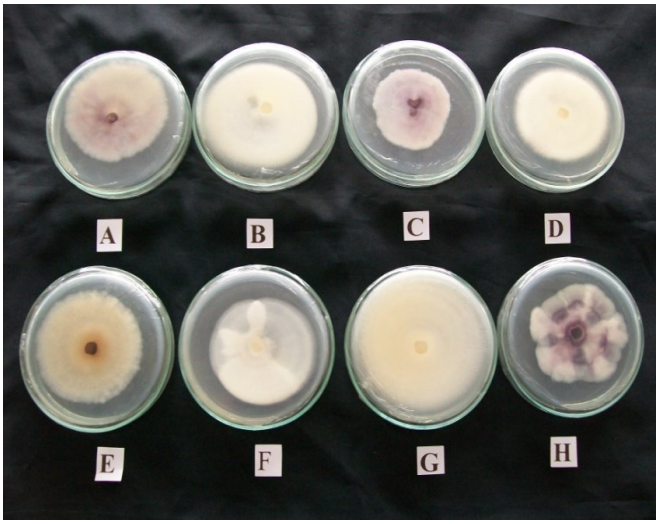


Figure 1. Isolate of *Fusarium oxysporum* f.sp. *cepae* from different cultivar and central areas of shallot production

Morphology of *Fussarium oxysporum* f.sp. *cepae* as a result of isolation from the plant tissue of shallot with moler symptom is presented in Figure 2. *Fusarium oxysporum* f.sp. *cepae* formed microconidium, macroconidium and chlamidsphora. Based on the observations in this study and the morphology of *F. oxysporum* described by Joffe (1986) and Leslie and Summerell (2006), this species had insulated hyphae, microconidium and macroconidium. The shape of microconidium was ellipse, having one or two cells, was not false head, was formed in a simple fialid or short lateral conidiophore and having a great number of it. Meanwhile, the shape of macroconidium was similar to *cano* having 3 *septas* with thin buttress, the apical cell was crooked, slightly hook and the basal cell section formed some cell similar to foot, about 25-30 x 4.0-4.5 µm. Chlamidosphora had thick and smooth buttress. It was formed intercalarily or terminal at the branch of short lateral from mycelium.

Identification based on structural morphology of asexual reproduction showed much variation on structural nature/characteristics. This fungus was put into Elegans section. Morphological isolation/splitting showed little difference and the variations depended much on its environment. Some specifications of host *(rhizome)* of each isolate was very limited. Furthermore, isolates and the same host (rhizome) or similar was determined as one specialist form (Kistler 1997).

VCG of Isolates of *Fussarium oxysporum* f.sp. *cepae*

This test was conducted to know whether the isolates of *Fussarium oxysporum* f.sp. *cepae* were obtained from the same group or not. Nit mutant of *Fussarium oxysporum* f.sp. *cepae* was obtained by inoculating the original breeding disk of fungus at a minimum level to turn the medium of chlorate (MCM) into minimum of the medium (MM). The colony of *Fussarium oxysporum* f.sp. *cepae* having thin and fast growth and

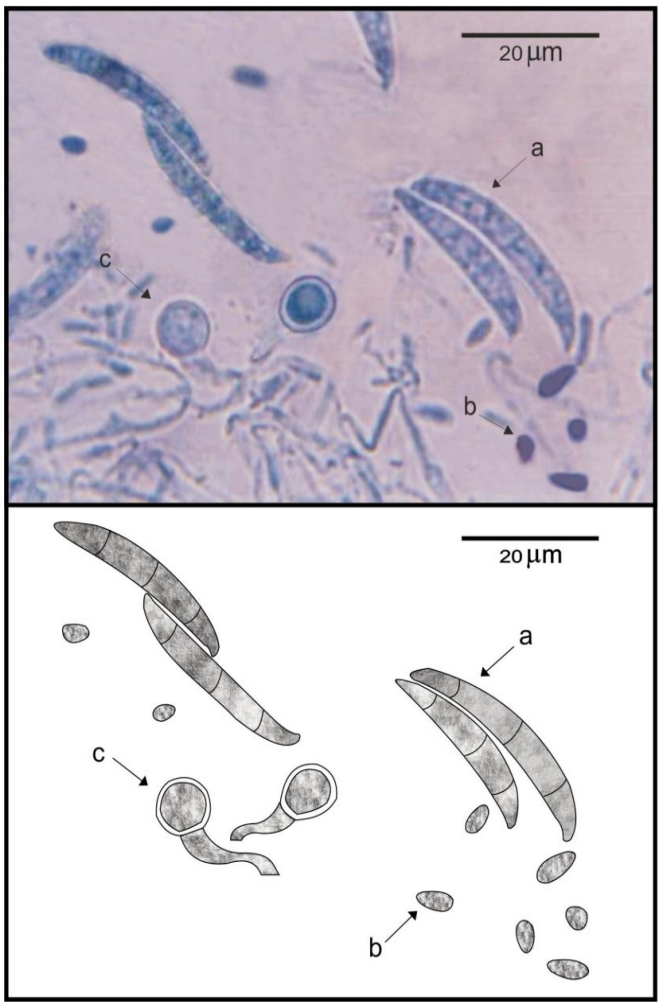


Figure 2. Morphology of *Fusarium oxysporum* f.sp. *Cepa* (a) macroconidium; (b) microconidium; (c) chlamidospora

did not form an air mycelium was said to be a nit mutant of *F. oxysporum* f.sp. *cepae* (Corell et al. 2002).

Furthermore, the test result showed that among the 8 isolates of *F. oxysporum* f.sp. *cepae* (Isolates A, B, C, D, E, F, G and H), only 4 of them (Isolates A, B, C and D) successfully formed nit mutant at the medium of MMS. Therefore, only these 4 isolates could be used as a vegetative test of compatibility group and that only these 4 isolates could be characterized. The nit mutant was marked by the thin mycelium formation at the medium of MM (Figure 3A). Meanwhile, for isolates that failed to form the nit mutant, the growth of mycelium at the medium of MM was thick (Figure 3B).

The complementation test was conducted by inoculating the breeding disk of the two nit mutants obtained from the different parent at about 1-3 cm using the medium of MM and incubated at room temperature for 7 d. The vegetative compatible nit mutant could make some complementation between each other by forming heterokarion at the medium of MM that could be observed from the thick and tight formation of mycelium where the colony of the two nit mutants touched (Correll et al. 2002).

Table 1. Vegetative compatible group from isolate of *Fussarium oxysporum* f.sp. *cepae* from Bantul (A, B, C), Brebes (D, E, F) and Nganjuk (G and H)

Fusarium oxysporum f.sp. cepae	*Fusarium oxysporum* f.sp. *cepae*							
	Isolate A	Isolate B	Isolate C	Isolate D	Isolate E	Isolate F	Isolate G	Isolate H
Isolate A	X	--	--	--	X	X	X	X
Isolate B	--	X	--	--	X	X	X	X
Isolate C	--	--	X	--	X	X	X	X
Isolate D	--	--	--	X	X	X	X	X
Isolate E	X	X	X	X	X	X	X	X
Isolate F	X	X	X	X	X	X	X	X
Isolate G	X	X	X	X	X	X	X	X
Isolate H	X	X	X	X	X	X	X	X

Note: -- No heterokarion formation; X: test of Vegetative Compatible Group was not conducted

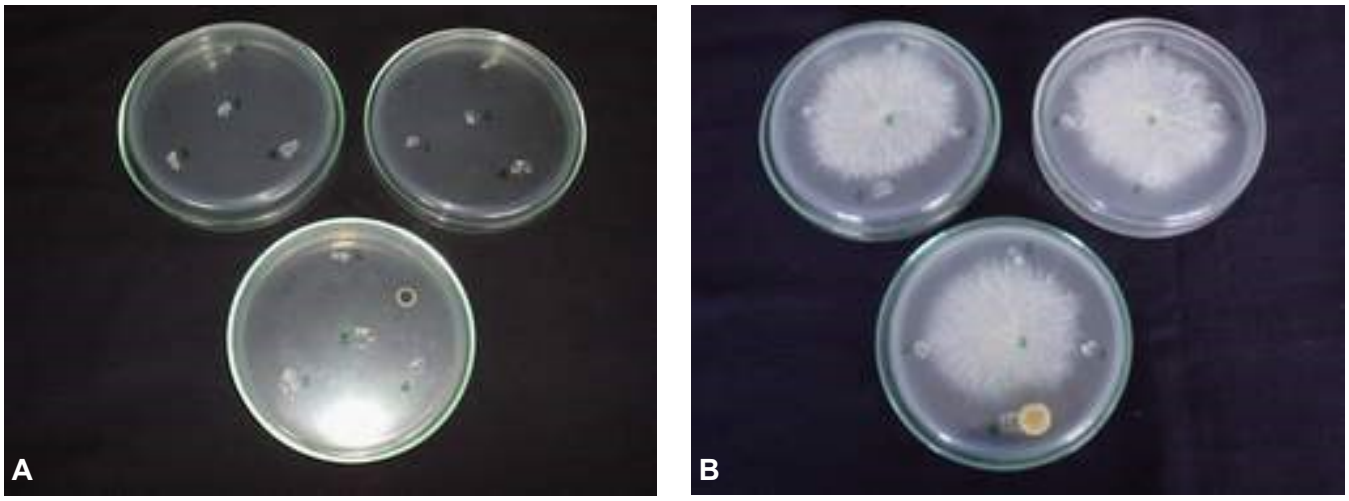


Figure 3. The Test Result of Vegetative Campatibility Group between *Fussarium oxysporum* f.sp. *cepae* of Isolates A,B, C, D (A) and Isolates of E, F, G, H (B).

Moreover, the observation result after 1 wk at the medium of MM showed that among the 4 isolates (isolates A, B, C, D), neither one was capable of forming heterokarion. This implies that genetically, these 4 isolates were different from one and another. This meant that each had different Ras (Table 1). Therefore, those 4 isolates could be characterized as the four different ras: isolate A, B, C and D as the Ras of 1, 2, 3 and 4.

CONCLUSION

Twisting disease occurred in the three central areas of shallot production: Kabupaten Bantul, Kabupaten Brebes and Kabupaten Nganjuk.

In those three central areas of shallot production, it was found that among the 8 isolates of *Fusarium oxysporum* f.sp. cepae, only 4 of them have different VCG. These four were characterized as the four of different Ras, isolate A, B, C and D and Ras 1, 2, 3 and 4.

ACKNOWLEDGEMENT

We would like to thank the Directorate General of Higher Education, Republic of Indonesia for funding this research and the staff of Plant Health Laboratory for their technical assistance.

LITERATURE CITED

Amstrong GM, Amstrong JK. 1981. *Formae specia-les* and Races of *Fusarium oxysporum* causing wilt disease. Pages 391- 399 in: Fusarium: disease, biology, and taxonomy. P.E. Nelson, T.A. Tonssom, and R.J. Cook, eds. Pennsylvania State University Press, University Park. 457 pp.

Correll JC, Klittich CJR, Leslie JF. 1987. Nitrate nonutilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. Phytopathology 77(12): 1640-1646

Correll JC, 1991. The relation between formae speciales, races, and vegetative compatibility groups in *Fusarium oxysporum*

Joffe AZ, 1986. Fusarium species: their biology and toxicology. John Wiley & Sons. New York. 588p.

Kistler HC, 1997. Genetic diversity in the plant-pathogenic fungus *Fusarium oxysporum*. *Phytopathology* 87(4): 474-478.

Leslie JF, Summerell BA. 2006. The *Fusarium* laboratory manual. Blackwell Publishing. Ames, Iowa.

Puhala JE. 1988. Classification of Strains of *Fusarium oxysporum* on the basis of vegetative compatibility. Can. J. Bot. 63: 179-183.

Wakman W. 2004. Medium dan Metode Isolasi Jamur *Fusarium* dan Patogen Tular-Tanah Lainnya. *Prosiding Simposium Nasional I Tentang Fusarium. Purwokerto.* 59 – 69.

Nelson PE, Toussoun TA, Cook RJ. 1981.Fusarium: disease, biology, and taxonomy. The Pennsylvania State University Press. University Park & London. 457p.

Wiyatiningsih S. 2012. Disease intensity of twisting disease in 3 area of shallot production center. Plumula. Journal of Agrotechnology. 1(1): 1-12.

Philippine Journal of Crop Science (PJCS) Special Issue 2016 41(1):41-48

Identification of Primary Nutrient Element Deficiency in Soybean Based on Leaf Features

Akhmad Fauzi*, I GedeSusrama MD, Sukendah, Teguh Soedarto, and Mohammad Idhom.

University of Pembangunan Nasional "Veteran" Jawa Timur, Surabaya, East Java,Indonesia
*Corresponding author, akhmadfauzi@upnjatim.ac.id

One of the problems in the cultivation of soybean is deficiency of nutrient elements. Deficiency can be indentified from the appearance of symptoms on the surface of the leaves. However, accurate identification requires expertise and some analysis. This study tried to create a quick method to identify nutrient element deficiency based on color features on the surface of the leaves. Deficiency of the major macro nutrients nitrogen, phosphorous, and potassium were identified using Classification Method of K-Nearest Neighbour (K-NN) and Artificial Neural Networks (ANN) Backpropagation. Since soybean leaf are usually green, the features that can be used is green color from RGB color's section (Red, Green, Blue). Characteristics used in the study were: average intensity, smoothness, entropy, five of moment invariant, energy, and contrast. Identification and classification were conducted on three primary nutrient elements i.e. potassium, nitrogen, and phosphorous using 30 samples for each nutrient. The results showed that the identification of nutrient element deficiency can be done by Classification Method of K-Nearest Neighbour (K-NN) and Artificial Neural Networks (ANN) Backpropagation. The accuracy of K-Nearest Neighbour was 69.44% and 77.78 % for Artificial Neural Networks Backpropagation.

Keywords: ANN Backpropagation, K-Nearest Neighbour, nutrient elements, soybean

INTRODUCTION

Soybean (*Glycine max* L. Merrill) is an annual plant that generally grow upright. Aside from soil physical conditions, another very important factor to consider in soybean growth is the fertility of the soil. Soil fertility levels are influenced by the content or adequacy of nutrients in the soil. As with other plants, according to Adisarwanto (2000) soybeans also require macro and micronutrients. Three macro nutrients; nitrogen (N), phosphate (P) and potassium (K); are needed in large quantity by almost plants. Nitrogen deficiency reduced shoot growth in pistachio (Afrousheh etal. 2007). The characteristic deficiency sympton of N is the appearance of uniform yellowing of leaves including the vein. In dicotyledonous crops like soybean the leaves detach easily under extreme deficiency. Phosphorous is a limiting factor for crop yields on over 30% of arable land on the planet (Vance et al. 2003). Deficiency of phosphorous decreased total leaves and roots of *Vigna subterranea* (Temegne et al. 2015). Potassium is regarded as one of the major nutrient which affects the yield and quality of grain and fruits (Ruiz and Romero 2002).

Interpreting visual nutrient deficiency symptoms in plant can be difficult because: (1) many symptoms appear similar - nitrogen and sulphur deficiency symptoms can be very alike; (2) multiple deficiencies can occur at the same time; (3) crop species or some cultivars differ in their ability to adapt to nutrient deficiencies; (4) hidden hunger - plants may be nutrient deficient without showing visual clues; (5) pseudo (false) deficiency symptoms (McCauley et al. 2011). The simple and inexpensive available tool to identify nutrient deficiency symptoms based on pictures requires expertise and some analysis. This study offered alternative method using artificial visual system, allowing the nutrient disorder at earlier stages in soybean. Artificial visual system is a computer system in which a set of methods and techniques are able to interpret or asses images automatically or semiautomatically (Punam and Udupa 2001). It has been used in maize to identify magnesium (Mg) deficiency (da Silva et al. 2014). Sena et al. (2008) found that artificial visual technique was better than a portable chlorophyll meter (SPAD) to evaluate the difference of three nitrogen levels on wheat (*Triticum aestivum* L.).

Knowing the important role of nitrogen, phosphorous and potassium on plant growth and yield, this work proposed the use of Artificial Visual Analytic System to identify the deficiency symptoms of the three macro nutrients based on the leaf's features. The system has been developed using soybean grown hydroponically in greenhouse.

MATERIALS AND METHODS

Experimental Design and Treatment

The study was conducted at the Faculty of Agriculture's greenhouse UPN "Veteran" East Java,

using a hydroponic system (growing soyben without soil using mineral nutrient solution in a water solvent to control the nutrient uptake).

In this research, growing soybean by hydroponic was done using a hollow pipe. A system for growing soybean using hydroponic technique which meets the requirement for adequate light, oxygen and water circulation was developed. The technique include the following: (1) Seed selection from a quality, healthy and pest-free parent; (2) Preparation of planting media using pipes designed for hydroponics cultivation. Planting media can be a mixture of heat-sterilized husk and sand pebbles; (3) Transplanting seedlings to a growing media taking care not to damage the roots formed, then watering the plants to provide moisture; (4) Set-up the hydroponic cultivation technique. This hydroponic technique is much easier and simpler than conventional soybean cultivation where crop management practices are routinely done such as pruning, weed cleaning, and so on. Despitethe risk of pests and disease attacks on low hydroponic plants, available labor is sufficient just for fertilizer application and irrigation done at the beginning of planting, 25 days after f planting, and at 40-45 days after planting; 5) harvesting which starts when the plants are 2.5 to 3-month old or when the leaves and pods turned yellow.

Conventional cultivation of soybean, applies urea (N = 46%), ZA (N = 21%), KNO3, NPK and high N content fertilizer to correct N deficiency. For the treatment of phosphorus deficiency, SP36 (P = 36%) fertilizer, NPK, MKP and high content P fertilizer are applied. Potassium deficiency is treated by adding KCl (K = 52%) fertilizer, NPK, MKP, and high K content fertilizer. (Keinoet al. 2015). Phosphorus deficiency was characterized by the color of the lower part of the leaves, especially the red leafy bones, curved, and twisted (distorted) leaves. Leaves, branches and stems are also purple. Lack of this element retards root growth and affects flower formation. treatment of Soybean plants deficient in potassium are characterized by shrinking of the leaves, especially the older leaves. The edges of the leaves turn yellow, then brown and eventually die and fall off the plant.The fruits formed are small and imperfect. The soybean variety used in this experiment was Wilis. The amount of NPK fertilizer applied was 2 g Urea, 0.5 g SP36 and 0.25 g KCL L^{-1} water.

Single factor experiment consisting of pots with minus one (pots 2, 3, 4) and minus two (pots 5, 6, 7) nutrient elements was arranged in CRD (Table 1). Two-wk old soybean plants were transferred in fertilized pots and maintained until the end of the vegetative stage. After which, plant samples were harvested, oven-dried at 70 C for 24 hr, then the dry weight was measured. Total N content of plant tissue was analyzed by Kjeldhal method, exchangeable K by NH4OAc method, and available P by Bray II method. Soil pH was measured following the electrometric (pH: H2O) method. The results of the 2-month cultivation of soybean (Figure 1) were used as basis for the dataset (test data).

Table 1. Summary of the experimental treatment of nirogen, potassium, and phosphorous

PI Treatment	Nitrogen	Potassium	Phosphate
1	Recommendation	Recommendation	Recommendation
2	Without	Recommendation	Recommendation
3	Recommendation	Without	Recommendation
4	Recommendation	Recommendation	Without
5	Without	Without	Recommendation
6	Without	Recommendation	Without
7	Recommendation	Without	Without

Image Texture Analysis

Image texture analysis is divided into 3 parts: first is a statistical approach, second is moment invariant, and the last is the co-occurrence matrix.

Statistical approach

Statistical approaches are commonly used approach for texture analysis based on histogram statistical intensity properties. One class of measurement is based on the statistical moment. Calculation of the *n*th moment about the mean is given by:

$$\mu_n = \sum_{i=0}^{L-1} (z_i - m)^n \, p(z_i) \qquad (1)$$

Where z_i i is a random variable indicating intensity, p (z) is the intensity level histogram in the region, L is the number of intensity levels available, the mean (average) intensity calculated by the formula:

$$m = \sum_{i=0}^{L-1} z_i \, p(z_i) \qquad (2)$$

Moment invariant

The size of the relative smoothness of the intensity in the region. R is 0 for the region in a constant intensity and close to 1 for the region with a large excursion in the level of intensity. In practice, the variant used in this measure is normalized in the range [0,1] by division by (L-1) 2. Smoothness was calculated using the formula:

$$R = 1 - 1/\left(1 + \sigma^2\right) \qquad (3)$$

Where σ is the size of the standard deviation measured using the formula:

$$\sigma = \sqrt{\mu_2(z)} \qquad (4)$$

Entropy is used to measure the randomness of the image intensity values, calculated by the formula:

$$e = \sum_{i=0}^{L-1} p(z_i) \log_2 p(z_i) \qquad (5)$$

Moment Invariants 2-D Moment of order (p + q) on the digital image f (x, y) is defined as :

$$m_{pq} = \sum_x \sum_y x^p y^q f(x, y) \qquad (6)$$

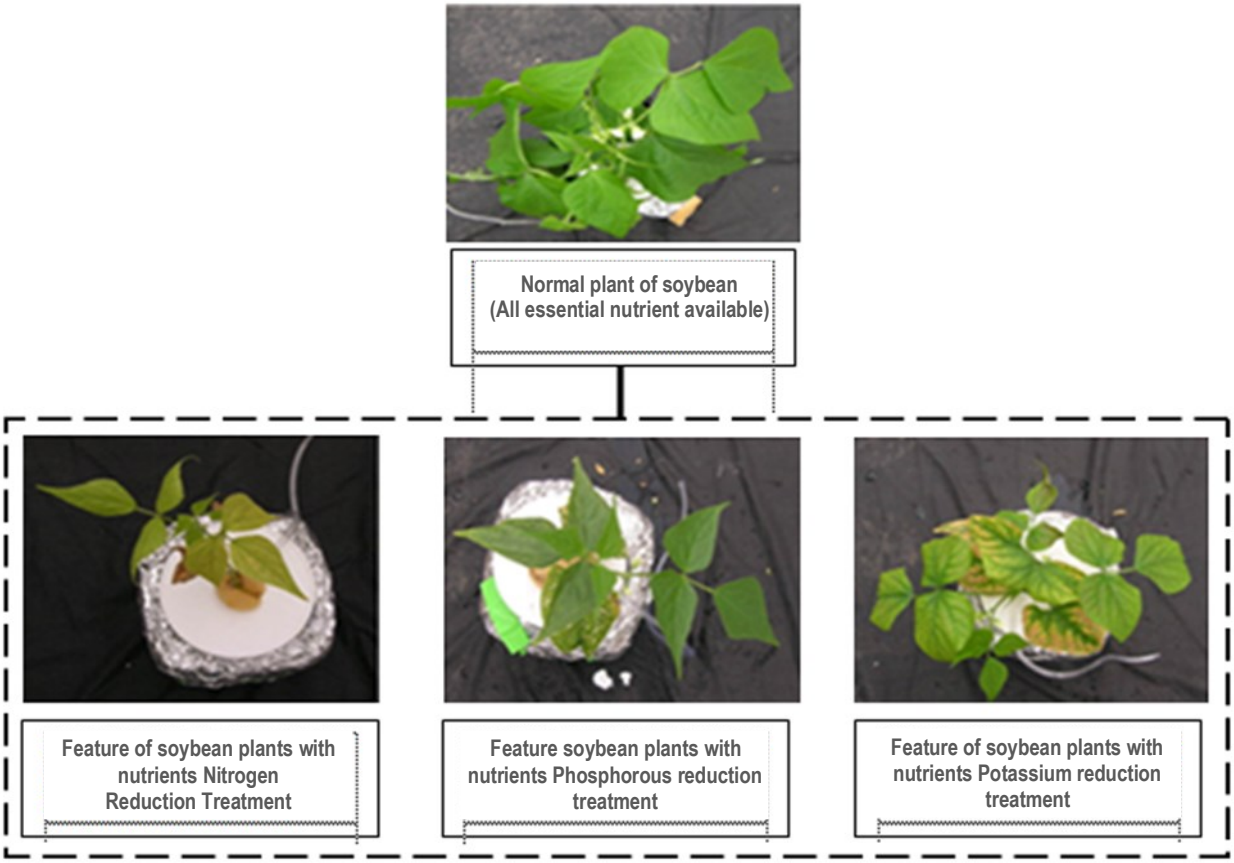


Figure 1. Plants from each of the fertilizer treatment used to obtain the test data and training data

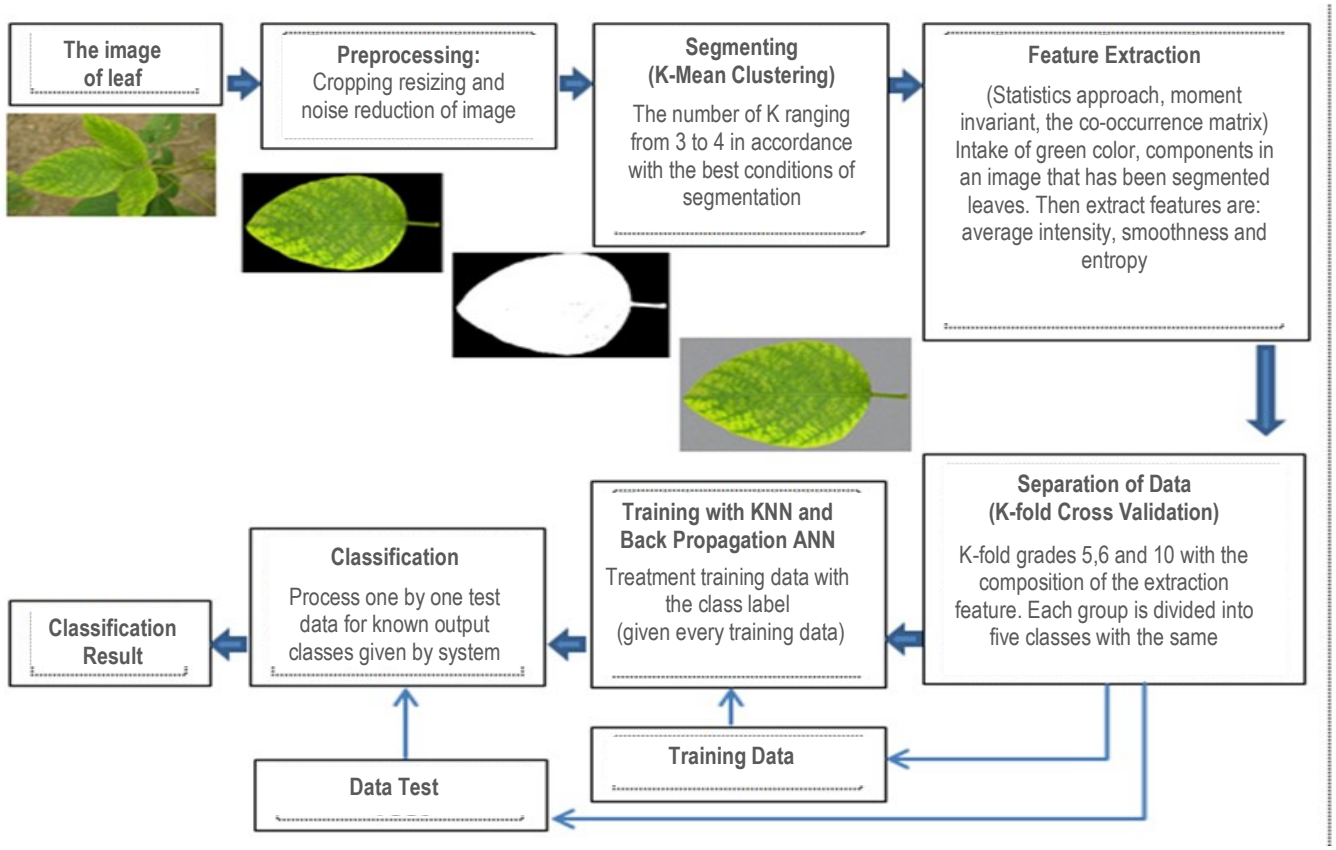


Figure 2. Artificial visual analytic system of primary nutrient element deficiency in soybean

For p, q = 0, 1, 2, ..., where the summation over the spatial coordinates x and y are stretching the image. The related central moments are defined as:

$$\mu_{pq} = \sum_{x}\sum_{y}(x-\bar{x})^{p}(y-\bar{y})^{q} f(x,y) \quad (7)$$

where :

$$\bar{x} = \frac{m_{10}}{m_{00}} \quad (8)$$

$$\text{dan } \bar{y} = \frac{m_{01}}{m_{00}} \quad (9)$$

For p, q = 0, 1, 2, …, where :

$$\eta_{pq} = \frac{\mu_{pq}}{\mu_{00}^{\gamma}} \quad (10)$$

Normalized central moment from order (p + q) is defined as:

$$\gamma = \frac{p+q}{2} + 1 \quad (11)$$

For p + q = 2, 3, ... A number of seven invariant moments are not sensitive to translation, scale change, reflection, and rotation can be derived from the following equation:

$$\phi_1 = \eta_{20} + \eta_{02} \quad (12)$$

$$\phi_2 = (\eta_{20} - \eta_{02})^2 + 4\eta_{11}^2 \quad (13)$$

$$\phi_3 = (\eta_{30} - 3\eta_{12})^2 + (3\eta_{21} - \eta_{03})^2 \quad (14)$$

$$\phi_4 = (\eta_{30} + \eta_{12})^2 + (\eta_{21} - \eta_{03})^2 \quad (15)$$

$$\phi_5 = (\eta_{30} - 3\eta_{12})(\eta_{30} + \eta_{12})[(\eta_{30} + \eta_{12})^2 - 3(\eta_{21} + \eta_{03})^2] + (3\eta_{21} + \eta_{03})(\eta_{21} + \eta_{03})[3(\eta_{30} + \eta_{12})^2 - (\eta_{21} + \eta_{03})^2] \quad (16)$$

$$\phi_6 = (\eta_{20} + \eta_{02})[(\eta_{30} + \eta_{12})^2 - (\eta_{21} + \eta_{03})^2] + 4\eta_{11}(\eta_{30} + \eta_{12})(\eta_{21} + \eta_{03})$$

$$\phi_7 = (3\eta_{21} + \eta_{03})(\eta_{30} + \eta_{12})[(\eta_{30} + \eta_{12})^2 - 3(\eta_{21} + \eta_{03})^2] + (3\eta_{12} + \eta_{30})(\eta_{21} + \eta_{03})[3(\eta_{30} + \eta_{12})^2 - (\eta_{21} + \eta_{03})^2] \quad (17)$$

Co-occurrence Matrix

Intensity of co-occurrence matrix is a matrix that describes the frequency of appearance of two pairs of pixels with certain intensity within a certain distance and direction in the image. Intensity of co-occurrence matrix p (i1, i2) is defined with two simple steps. The first step is to determine the distance between two points in the vertical and horizontal [vector d = (dx, dy)], where the quantities dx and dy are expressed in pixels as the smallest unit in digital image. The second step is to calculate the pair of pixels that have intensity values i1 and i2 and is in the pixels in an image. Then the results of each pair of intensity values are placed on a matrix corresponding with its coordinates, where the abscissa of intensity values of i1 and to the coordinate for the i2 intensity values. Energy in the co-occurrence matrix is a feature to measure the intensity of the pair concentration on co-occurrence matrix, and is defined with (Haralick et al. 1973).

$$Energi = \sum_{i1}\sum_{i2} p^2(i_1, i_2) \quad (18)$$

The classification method k-Nearest Neighbour (k-NN)

K-Nearest Neighbour method (k-NN) is a method for the classification of objects based on data learning that was located closest to the object. The data of learning is projected onto a lot space dimension, where each dimension represents a feature of the data. The space is divided into sections based on data classification learning. Distance from neighbour is usually calculated based on Euclidean distances with the following general formula (Parvin et al. 2008; Bhatia 2010):

$$d = \sqrt{(a_1 - b_1)^2 + (a_2 - b_2)^2 + \cdots + (a_n - b_n)^2} = \sqrt{\sum_{i=1}^{n}(a_i - b_i)^2} \quad (19)$$

K-NN classification is done by searching k-pieces nearest neighbour and choose grade with highest ki in the class ω_i. The steps for k-NN classification is as follows (Altman 1992; Bhatia 2010):

1. If a set vectors training data have N data points as a whole, then get to know the k-pieces nearest neighbours of sample x where k is an odd number.
2. Of these k-pieces nearest neighbour, identify the number of ki vectors in class ωi, i = 1,2, ..., M with Σiki = k.
3. Insert on ωi class with a maximum value of ki.
4. If two or more ωi classes have E nearest neighbours, then there was a balanced condition (conflict) and conflict-solving strategies used.
5. For each of the classes involved in the conflict, determine the distance between the x with ωi class based on E nearest neighbour found in the ωi class.
6. If the m training patterns of ωi class involved in the conflict indicated by yim = {y1im, ..., ynim} then the distance between the x with class ωi is:

$$d_i = \frac{1}{E}\sum_{j=1}^{N}\left|(x_j - y_j^{im}\right|$$

Insert x into the class with the smallest distance, which is x ∈ωC, if dC<di for i, C ∈ [1, ..., M] and i ≠ C.

Figure 3 is an example of k-NN sorting rule. Figure 3 (a) shows that the number of nearest neighbour k = 3 that are inside the circle is not a conflict between class a and class b so it does not need strategies of conflict resolution and determined that these test points get into the a class. While for Figure 3 (b) there is a

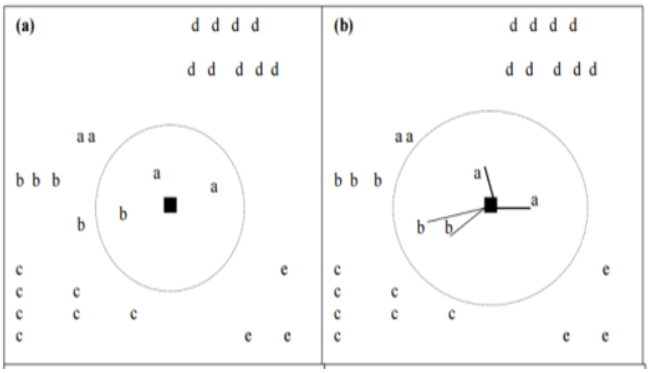


Figure 3. Illustration of k-NN sorting rule. (a) the early model of k-NN; (b) k-NN models with strategies of conflict resolution (Data Anayzed 2016).

conflict between class a and class b by the number of nearest neighbour k = 4 inside a circle that has the number of nearest neighbour E for each class is 2. Subsequently conflict resolution rules such as the k-NN classification rule above by calculating the shortest distance between class a and b to be prescribed by the test point included in the class a or b (Sing 1999).

RESULTS AND DISCUSSION

Nutritional deficiency is a major problem in the growth and yield of most crop plants. Therefor, providing a method to identiy the symptom as soon as posibble to overcome this problem is very important. The present research developed a tool to quickly detect nutrient deficiency based on leaf feature using K-Nearest Neighbour (K-NN) and Artificial Neural Networks (ANN) Backpropagation. This tool is a system consisting of 6 stages as shown in Figure 3. The first step was preprocessing stage to do cropping, resizing and noise reduction in the image. The second step was segmentation using K-means clustering method with the number of k from 3 to 4 in accordance with the best conditions for a given segmentation results. The third stage was retrieval of green on the color components of leaf image which are already segmented. Then the features extracted were: average intensity, smoothness, and the entropy of a statistical approach; 5 of 7 moment invariants; and energy and contrast of co-occurrence matrix approach. Next is the separation of data from the sample image data which is divided into two groups: training data and test data. The composition was used to adjust analytical results with the technique of K-fold cross validation. Training data was generated using K -Nearest Neighbour (K-NN) and Artificial Neural Networks (ANN) Backpropagation. The last stage was classification. This step was done by processing test data one at a time to know the class output given by the system.Then the results were matched with the real class to know the insurance system in doing the classification.

The artificial visual analytic system in Figure 3 was developed using image analysis with three approaches: a statistical approach, moment invariance, and co-occurrence matrix. Statistical analysis of principal component analysis (PCA) obtained 10 features as shown in Table 2.The texture parameters used were: i) average intensity, ii) smoothness, iii) entropy; iv) 5 components of moment invariants, v) energy, and vi) contrast. Image analysis gave features including smoothness, and the entropy value of the intensity of green colour in the leaves. Moment invariant analysis showed there were 5 observed data and these were 1, 2, 4, 6, and 7. While the co-occurrence matrix approach provided an alternative image analysis technique of the statistical approach. The features extracted from this matrix were energy and contrast. Energy to measure the constancy of energy's intensity and contrast to measure the degree of differences on the intensity of leaf green colour.

Table 2 describes how verification of image analysis was done by K-fold Cross Validation Technique. The result showed that the error rate of K-fold value from 5 to 10 was 0.0833, 0.1000, 0.1167, 0.1333, 0.1500, and 0.1667, respectively. The smallest error rate was obtained when the K-fold value is 5. Composition data for training and test with K-fold value of 5, 6 and 10 were 48,12; 50,10; and 54,6, respectively. The validation testing was done using training data as test case on the system, then used test data as input data for classification. The training parameters used were the target error 0.001 using the criteria of Sum Squared Error (SSE), the training rate of 0.2, momentum 0.95, and the maximum number of iterations was 5000 times. A total of 10 features from each of the 12 test images for K-fold 5 were subjected to classification test data with K-NN method and ANN Backpropagation. For ANN Backpropagation, it used 1 hidden layer with 150 neurons, while the output layer used 1 neurons. The results of test data classification by the system were compared with actual test data (manual).

The accuracy of classification system of nutrient deficiency on nitrogen, phosphorous and potassium based on K-NN is shown in Table 3. The average value of accuracy percentage was 69%. This value was obtained from comparing test data which were classified correctly with all tests data. Meanwhile, the accuracy of classification system based on ANN Backpropagation was 77.78%.

Recently many image processing techniques are developed to identify nutritional status and to classify plant leaf diseases or other uses. Treder et al. (2016) showed that using image analysis to estimate the nitrogen nutrient status of apple trees was better than leaf greeness meters. Fauzi et al. (2016) used image analysis for energy conservation to manage application Virtual Machine. Ghaiwat and Arora (2014) discussed some classification techniques such as k-Nearest Neighbor Classifier, Probabilistic Neural Network, Genetic Algorithm, Support Vector Machine, Artificial Neural Network, and Fuzzy Logic. According to reports selecting a classiication method is always a difficult task because the quality or results can vary for

Table 2. Test data of 10 features of soybean leaf

Test Data	Features									
	RTA	SMO	ENT	M1	M2	M4	M6	M7	ENE	KON
1	29.1729	0,0155	34,826	55,427	116,998	229,311	290,055	448,459	0,2786	677,212
2	33.9193	0,0230	33,328	57,097	119,975	217,963	285,323	447,507	0,3116	843,237
3	29.0943	0,0156	35,126	55,885	118,344	245,504	307,731	483,301	0,2784	693,236
4	29.0359	0,0148	34,111	55,716	118,278	243,760	309,883	476,693	0,2721	786,404
5	30.0713	0,0158	35,678	55,993	118,586	242,964	304,569	480,587	0,2659	696,455
6	36.568	0,0159	35,851	56,038	118,700	239,468	300,445	476,500	0,2584	685,670
7	35.2274	0,0153	42,650	54,728	115,021	232,160	299,284	458,194	0,1738	837,451
8	34.4043	0,0144	42,666	54,251	113,855	232,463	296,159	461,905	0,1704	860,139
9	27.2064	0,0103	37,283	53,438	112,981	220,147	297,404	431,424	0,2050	689,388
10	26.4059	0,0102	36,640	53,492	113,047	213,572	275,919	425,218	0,2204	570,943
11	27.9246	0,0100	39,025	53,477	113,233	212,033	273,976	423,380	0,1850	635,500
12	30.2173	0,0111	40,013	54,143	114,539	213,308	274,956	425,375	0,1755	598,338

Table 3. The accuracy percentage of the classification of nutritional deficiency in soybean by K-NN (K-Nearest Neighbor)

Test Data	Classification Results K-NN (K-Nearest Neighbor)			Target Class	Category
	1-NN	3-NN	5-NN		
1	2	2	0	2	Potassium
2	2	2	2	2	
3	2	2	2	2	
4	0	2	2	2	
5	1	0	0	1	Phosphorous
6	0	0	0	1	
7	1	1	1	1	
8	1	1	1	1	
9	1	1	0	0	Nitrogen
10	1	0	1	0	
11	0	0	0	0	
12	0	0	0	0	
Correct results	8	9	8		
Accuracy (%)	66.67	75.00	66.67		
Average (%)	69.44				

Table 4. The accuracy percentage of the classification of nutritional deficiency in soybean by Artificial Neutral Networks (ANN) Backpropagation

Test Data	Classification ANN (Artificial Neural Networks) Back propagation			Target Class
	Test 1	Test 2	Test 3	
1	2	2	2	2
2	2	2	2	2
3	2	2	2	2
4	2	1	2	2
5	1	1	1	1
6	1	1	1	1
7	1	2	2	1
8	0	1	1	1
9	1	1	1	0
10	0	0	0	0
11	0	0	0	0
12	0	0	0	0
Correct results	10	8	10	
Accuracy (%)	83.33	66.67	83	
Average (%)		77.78		

different input data. In this research the system for identifying the symptoms of nutrient deficieny on nitrogen, phosphate and potassium comprised of 6 stages. There are many combination steps developed by numerous researchers. One was developed by Keskar et al. (2013) which consisted of 4 stages.

Based on accuracy percentage, ANN Backpropagation was better than K-NN to identify nutrient deficiency symptons of nitrogen, phosphorous and potassium in soybean. According to Kaur and Laxmi (2016), the advantages of ANN is that it is tolerant of noisy inputs and on instances classified by more than one output. On the other hand, Backpropagation Network is easy to implement, applicable to wide range of problems and able to form arbitrarily complex nonlinier mappings. Ghaiwat and Arora (2014) mentioned that K-NN is sensitive to irrelevant inputs (parameters). To support accurate and automatic detection of leaf diseases, Bashish et al. (2011) used the Neutral Network (NN) classifier that is based on statistical classification with a precision of around 93%.

The precision of the K-Nearest Neighbor (K-NN) and Artificial Neural Network (ANN) Backpropagation on determining leaf features of the primary nutrient deficiencies could still be enhance above 90% by making corrections to the inputs, including the leaf shape feature and leaf age. This system is still working offline, where the classification is done outside of the image-making process. Media should be integrated with the camera so that the system can work online and real time. Approach to other methods is also important to note considering that K-NN method and ANN Backpropagation also have weaknesses (Kaur and Laxmi 2016).

CONCLUSION

1. Identification of nutrient defficiency on nitrogen, phosphorous, and potassium in soybean based on leaf features could be done by Artificial Visual Analytic System with 6 stages.

2. The average precision of K-NN classifier was 69.44 % and ANN Backpropagation classifier was 77.78%.

3. The Artificial Visual Analytic System could still be improved by making corrections to the input parameters such as leaf shape and age.

ACKNOWLEDGEMENT

This research was funded in part by the Directorate General of Higher Education Republic of Indonesia and Informatic Laboratory UPN "Veteran"Jawa Timur. The Assistance of Sugiyanto, M Syahrul Munir, and Tri Lathief Mardi.

REFERENCES

Adisarwanto T, Suhartina. 2000. Determination of P Fertilizer Recommendation Based on Soil Tests in Entisol. Increasing Efficiency of Nutrient, Water and Land Management for Soybean in Entisol and Oxisol Paddy Fields. Balitkabi Final Report 1999/2000 Pp 1-15.

Altman, NS. (1992). "An introduction to kernel and nearest-neighbor nonparametric regression". The American Statistician. 46 (3): 175–185. doi:10.1080/00031305.1992.10475879.

Afrousheh M, Ardalan M, Hokmabadi H, Hatami S. 2007. Visual Deficiency Symptoms of Nitrogen, Iro, Magnesium, Manganese and Molybdenum (Macro-and Micronutrients) on Pistachio Seedlings. Middle Eastern and Russian Journal of Plant Science and Biotechnology, Vo.1 (2):61-65.

Bashish AD, Braik M, Ahmad SB. 2011. Detection and Classification of Leaf Disease Using K-mean Based Segmentation and Neutral Network based Classification. International Technology Journal, ISSN 1812-5638 : 267-275.

Da Silva FF, Luz PHC, Romualdo LM, Marin MA, Zuniga AMG, Herling VR, Bruno OM. 2014. A Diagnostic Tool for Magnesium Nutrition in Maize Based on Image Analysis of Different Leaf Sections. Crop Science. Vol 54: 738-745.

Fauzi A, Mulyadi E, Idhom M, Fadil A. 2016. Management of Virtual Machine as A ConservationEnergy System Private Cloud Computing. Proceeding International Seminar, MATECWeb of Conferences (58) p. 3008.

Ghaiwat SN, Arora P. 2014. Detection and Classification of Plant Leaf Diseases Using Image Prosessing Techniques: A Review. International Journal of Recent Advances in Engineering and Technology, Vol 2 (3): 1-7.

Haralick RM, Shanmugam K, Dinstein IH. 1973. Textural features for image classification.IEEE Transac, SMC-3:610-621.

Kaur L, Laxmi V. 2016. A Review on Plant Leaf Classification and Segmentation. International Journal of Engineering and Computer Science. Vol 5 No 8 : 17658-17661.

Keskar PV, Masare SN, Kadam MS, Deoghare SU. 2013. Leaf Disease Detection and Diagnosis. International Journal of Emerging Trends in Electrical and Electronics Vol. 2 Issue 2.

Keino L;, Baijukya F,Ng'etich W, OtingaAN. 2015. Nutrients Limiting Soybean (glycine max l) Growth in Acrisols and Ferralsols of Western Kenya.Author Information; Okalebo, John R; et al. PLoS One; San Francisco 10.12 (Dec 2015).

McCauley A, Jones C, Jacobsen J. 2011. Plant Nutrient Functions and Deficiency and Toxicity Symptoms. Nutrient Management Module, Montana State University No.9: 1-16.

Punam KS, Udupa JK. 2001. Optimum Image Tresholding via Class Uncertainty and Region Homogenity. IEEE Trans.Pattern Anal. Mach. Intell, 23:689-706.

RuizJM, Romero L. 2002. Realtionship between Fertilization and Nitrate Assimilation and Fruits Cucumber. Annals of Applied Biology, Vo. 140:241-245.

Sena DG, Pinto FDADC, De Queiroz, Santos NT, Khoury JK. 2008. Machine Vision Techniques and Multivariate Classifiers for Nitrogen Fertilization Doses Discrimination in Wheat. Enginering Agriculture, 28:187-195.

Singh S, Haddon J, Markou M. 1999. Neighbour Strategies For Image Understanding. Proc. Workshop on Advanced Concepts for Intelligent Vision, Systems (ACIVS'99), Baden-Baden, (2-7 August, 1999).

Tamer H, Kasem MA, Schmitt FG. 2016. Smoothness Indicators For Weno Scheme Using Undivided Differences. Proceedings of ALGORITMY 2016 pp. 155–164.

Temegne CN, Taboula JM, Nbendah P, Youmbi E, Taffouo EM, Ntsefong GN. 2015. Effect of phosphate deficiency on Growth and Phosphate Content of Three Voandzu (Vigna subterranea L.) Varieties. Journal of Agriculture and Veterinary Science, Vol 8 (9): 52-59.

Treder W, Klamkowski K, Kowalczyk W, Sas D, Wojcik K. 2016. Possibilities of using image analysis to estimate the nitrogen nutrition status of apple trees. Zemdirbyste-Agriculture, Vol 103 (3): 319-326.

Vance CP, Uhde-Stone C, Allan DL. 2003. Phosphorus Acquisition and Use: Critical Adaptations by Plants for Securing a Nonrenewable Resource. New Phytology, 157: 423-47.

GUIDELINES FOR CONTRIBUTORS TO THE PHILIPPINE JOURNAL OF CROP SCIENCE

General

The Philippine Journal of Crop Science (PJCS) is open to papers within the field of crop science and technology. Crop-related fields may include: agroforestry, biochemistry, biodynamic farming, biofuels, biotechnology, breeding, communication, culture, cytology, ecology, economics and policy, engineering, extension, genetics, management, marketing, morphology, physiology, postharvest handling, processing, production, protection, seed technology, soils and nutrient management, sustainable agriculture, systematics, teaching, and utilization.

Each article must be a report of original research which has not been previously or simultaneously submitted or published elsewhere, except as an abstract of an oral or poster presentation. Contributions must be in English language and submitted as a Major Paper, a Research Note or a Variety Registration. Review articles may also be published only if they summarize information in a field in which the literature is scattered or they treat published data or information so as to provide a new approach or stimulate further research.

It is encouraged that authors cite articles that have been published in the journal to increase the citation index of the journal.

Submit one hard copy and electronic copy of the manuscript to:

Dr. Rhodora R. Aldemita
Editor in Chief
The Philippine Journal of Crop Science
Institute of Crop Science, College of Agriculture and Food Science
University of the Philippines Los Baños
4031 College, Laguna, Philippines
Email address: pjcs.uplb@up.edu.ph

To ensure rapid and accurate publication, it is essential that manuscripts conform to the instructions below. Manuscripts which are not in accordance with the specifications and needs extensive editing will be returned to the authors.

Preparation of Manuscript

Each article, including tables, figures and other appendices should not be more than 30 pages using a letter paper (216 mm x 279 mm or 8.5" x 11"). Specifications for the preparation of the manuscript are:

***Spacing, Font Size and Margins*.** Type the manuscript in Microsoft Word Document (2000 or 2007 Version), in double space, and with 11-point Arial font. Margins should be 2.54 cm (1") on the top, bottom, and left- and right-hand sides of each page.

***Order and Contents*.** Arrange contents of the manuscript in the following order:

1. **Title** – The title of the article should be typed in uppercase letters, and should not be more than 15 words. Indicate the running title (not more than 40 letters) to be placed on the upper left-hand corner of each page;
2. **Author(s)' Name(s) and Address(es)** – Should be complete, with acronyms spelled out. Omit institutional and social designations, and indicate the corresponding author with email address;
3. **Keywords** – Should be in alphabetical order. List single word, compound or phrases that indicate major subjects, and concepts or ideas that are related or implied in the paper;
4. **Abstract** – Summarize the rationale, materials and methods, major findings, and conclusions in not more than 300-350 words;
5. **Introduction** – Should contain rationale, hypothesis, and objective(s) of the paper;
6. **Materials and Methods** – Should include details of the methodology for replicability, adoption or adaptation of the procedures for future studies as well as when and where the study was conducted. Cite if the procedure has been published elsewhere;
7. **Results and Discussion** – Results presented should be consistent with the objectives of the study. Discussion should be done for integration purposes, i.e. within the results of the study or with reference to other studies;
8. **Conclusion** – Should include the summary and implications of major findings of the study. Recommendations can be included or be stated under the heading "Recommendations";
9. **Acknowledgement** – Indicate the source(s) of funds and institutions or individuals who helped in the study;
10. **Literature Cited** – List literature cited in alphabetical order by main author's last name. Give complete information. Reference should be cited in the text by the last name of the author, (both authors when only

two; first author et al. when more than two) and year. Do not number literature cited. It is the author's responsibility to maintain the consistency between the in-text citations and the end list of literature cited. Examples of the end literature citations are:

Book – Taiz L, Zeiger E. 2006. Plant Physiology. 4th ed. Sunderland, Massachusetts: Sinauer Associates, Inc. 764 p.

Book chapter – Giller KE, McDonagh JF, Cadisch G. 1994. Can biological nitrogen fixation sustain agriculture in the tropics? In: Soil Science and Sustainable Land Management in the Tropics. Syers JK, Rimmer DL, editors. CAB International, Wallingford. p. 173-191.

Editor – Balasubramanian V, Ladha JK, Denning GL, editors. 1999. Resource Management in Rice: Nutrients. Dordrecht, The Netherlands: Kluwer Academic Publishers. 355 p.

Electronic references (end list).

Journal article from online database: Author. Year of publication. Title of the article. Title of the journal volume#(issue#):pages. Database name. <URL>. Date accessed.

Online book (electronic text or part of a book): Author. Date of publication. Title of the book. <URL>. Date accessed.

Web page: Author. Update date. Title of the document. Title of complete work. <URL>. Date accessed.

Journal article – Faustino FC, Garcia RN, Agtarap ML, Mendoza AMT, Lips SH. 2000. Salt tolerance in corn: Growth responses, ion accumulation, nitrate reductase and PEP-carboxylase activities. Philipp J Crop Sci. 25(1):17-26.

Organization as author – [DA-BAS] Department of Agriculture - Bureau of Agricultural Statistics. 2006. Crops Statistics of the Philippines 2001-2006. Diliman, Quezon City. 300 p.

Paper in a proceeding – Virmani SS. 2001. Opportunities and challenges of developing and using hybrid rice technology in the tropics. In*:* Peng SB, Hardy B, editors. Rice Research for Food Security and Poverty Alleviation. Proc. International Rice Conference. Philippines. 407 p.

Personal communications should not be included in the end list of literature cited but can be included in the text as – PJA Santos 2008 (personal communication) or (PJA Santos 2008, personal communication).

Thesis or Dissertation – Sastri AVB**.** 2006. Effects of co-solvents on the transesterification reaction time and reduction of free glycerin content of methyl ester produced from coconut oil. [MS Thesis] College, Laguna, Philippines: University of the Philippines Los Baños. 100 p. (Available at UPLB Library).

11. **Tables and Figures** – Number the tables and figures (graphs, Illustration and photographs) consecutively in Arabic numerals. Table should have a legend if necessary. Acronyms or abbreviations should be spelled out or defined in a footnote. Appropriate quantities and units in SI (*Système International*), and other qualifications should be indicated within the table or footnote to make the table a "stand alone" source of information. Letters in the table caption should be in Arial 10-point font (but can be reduced to a readable size if necessary). Figures with text should have Arial as the font style and must be at least 8 points or bigger. Graphs must have been generated preferably using the Excel program for uniformity. Microscopic illustrations should include magnification, preferably a bar denoting scale of measure.

    Tables and figures should conform to the page size and style of the journal. Figures or tables should be prepared as camera-ready copy and submitted as excel or JPEG files. They should measure exactly 16 cm (1890 pixels) or 8 cm (945 pixels) wide. The height, however, can be varied to the maximum size of 23 cm, including label.

    Electronic copy of picture must be submitted as JPEG picture files of at least 300 pixels per inch (PPI). Figures that do not meet this resolution requirement can have a poor printout. Paper submitted for initial assessment should have the tables, figures, photos/illustration incorporated in the text. During the final stage of paper preparations submit a separate file for the text document, tables and figures, photos/illustration. Do not incorporate tables in the text as this will be done by the PJCS.

Pagination. Number all pages of the manuscript consecutively at the right hand bottom corner of each page.

Numbers. Avoid beginning a sentence with a number. Spell out numerals from one to nine, except when followed by standard units of measure, and indefinite and approximate periods of time. Ordinal numbers should be treated as cardinal numbers (e.g. 8th,18th). Exceptions are numbers in tables, figures, graphs, and those in parenthesis.

Fractions. Spell out and hyphenate fractions (e.g. two-thirds). Exceptions are fractions in tables, figures, graphs, legends and those in parenthesis.

Units and Symbols. Use recommended SI units and symbols. Use exponents instead of slash (e.g. kg

ha^{-1}). Use words when unit of measure is not involved (e.g. grains per panicle). In the case of range of values, indicate the unit at the end of the range (e.g. 10-15 t ha^{-1}). Avoid beginning a sentence with a symbol. Use percent symbol (%) with figures only (e.g. 5%), and spell it out (percent) with written numbers. Indicate the US$ equivalent of other currencies at the first mention in the text, or indicate in a footnote in a table or a figure, if applicable.

Abbreviations and Acronyms. Spell out abbreviations and acronyms the first time they are used in the text (e.g. ATP for adenosine triphosphate, HYV for high yielding variety). Spell out abbreviations that are not generally known or specifically used in the paper at the first mention in the text. Commonly used abbreviations (e.g. ANOVA, DMRT, IQ, LSD, SD, etc.) need not be spelled out. For scientific names, shorten the generic name to the first capital letter followed by the species, by the 2nd time they are used. Spell out units of measure with five or less letters (when singular) as year, month, etc., except when preceded by a numeral from measurement. Abbreviate minute (min), second (s), hectare (ha), kilometer (km) and the rest, particularly for SI units. No period is needed at the end of each abbreviation and do not add "s" to the plural form.

The PJCS generally adheres to the Council of Science Editors (CSE) in Scientific Style and Format: The CSE Manual for Authors, Editors, and Publishers (7th ed, 2006).

Refereeing and Editing

Each manuscript submitted will be reviewed by two Referees. The Referees are asked to recommend to the Editors that a paper be accepted for publication, accepted after revision or rejected. A decision to accept the advice of the Referees to reject a paper is taken only after the paper has been read by two of the Editors. The Editors reserve the right to make alterations in manuscript submitted for publications. Such alterations will be made if manuscripts do not conform to the accepted scientific standards or if they contain matter which in the opinion of the Editors is unnecessarily verbose or repetitive. Papers that need extensive alteration will be returned to the Authors for checking and retyping. Unless such papers are returned within one month, they will be deemed to be new and given a revised date of receipt. The contents of the papers are the sole responsibility of the Author(s) and should not be construed to reflect the opinions of the Editor(s) and /or the Crop Science Society of the Philippines.

Papers should have undergone English editing and submitted to the journal with accompanying signed certificate by the English editor. Papers which still needed English editing will be charged accordingly

Proof and Reprints
Galley proofs are sent to the Authors for correction of printer's and similar errors. Avoid major alteration of the original text. Return proofs within 7 days. Reprints may be ordered at the Author's expense at the proof stage.

Publication and Review Fee
Upon acceptance of the paper for review, authors will be requested to pay a review fee of PhP2000 for CSSP member, PhP2500 for non-member and PhP 3000 for foreign authors.

For the publication fee, the journal will charge CSSP members a fee of PhP1000 per page for black and white and PhP2000 for colored pages. Non-members will be charged PhP1200 for black and white and PhP2500 for colored pages. Foreign authors will be charged PhP1400 per page for black and white and PhP3000 per page for colored pages. Authors will be provided with a printing invoice along with the pre-printing proof of their article for proof-reading and will be requested to pay half of the total publication fee before printing. Full payment must be made after the release of the journal.

The Philippine Journal of Crop Science

Published by the Crop Science Society of the Philippines and the
Federation of Crop Science Societies of the Philippines
Email: pjcs.uplb@up.edu.ph
Website: http://www.cssp.org.ph

Date _____

Co-author Agreement Copyright Transfer Form

(to be accomplish in duplicate)

In line with scientific integrity and publication ethics, we hereby submit the signatures of the authors of the paper entitled

_____
_____
_____, authored by
_____
_____
_____
_____

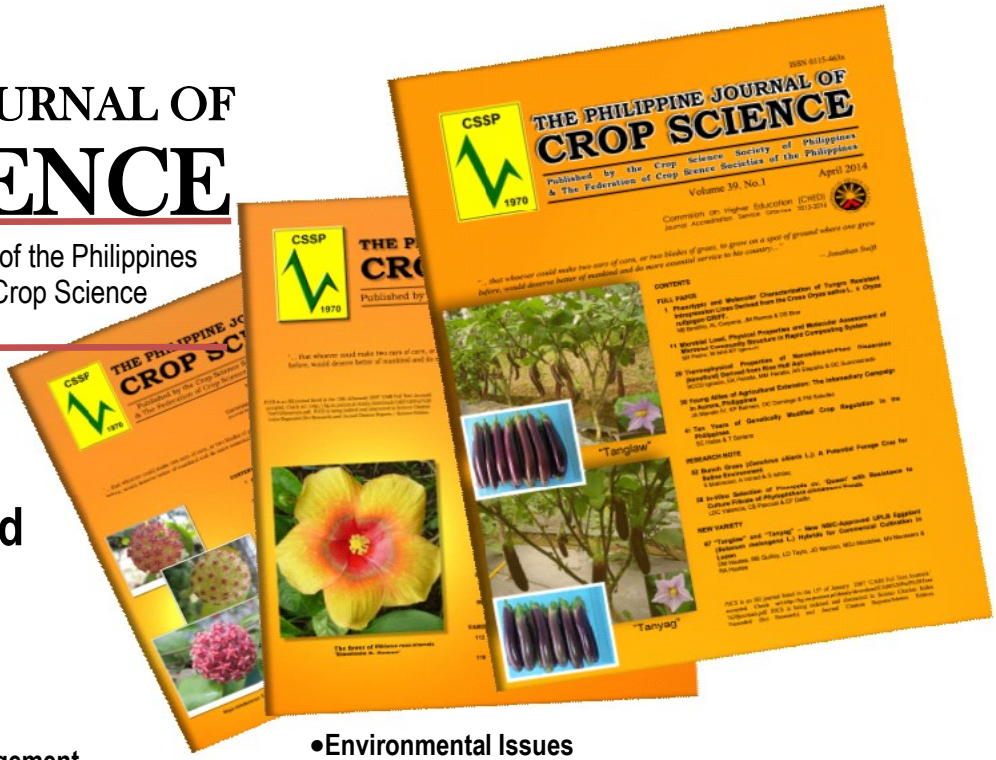
To the Philippine Journal of Crop Science editorial board.

Signing this form signify that we as joint authors are responsible for the content of the article as we are involved in the conduct and writing of the paper, and can answer queries and revisions if necessary. Our signatures also conform the transfer of copy-right to the journal once the article is accepted for publication.

_____	_____
Corresponding AUTHOR	**Senior AUTHOR**
(Signature over printed name)	(Signature over printed name)
_____	_____
AUTHOR	**AUTHOR**
(Signature over printed name)	(Signature over printed name)
_____	_____
AUTHOR	**AUTHOR**
(Signature over printed name)	(Signature over printed name)
_____	_____
AUTHOR	**AUTHOR**
(Signature over printed name)	(Signature over printed name)

THE PHILIPPINE JOURNAL OF CROP SCIENCE

Published by the Crop Science Society of the Philippines
And co-published by the Federation of Crop Science Societies of the Philippines



Get updated with the latest in agricultural researched in the Philippines

- Crop Production and Management
- New Varieties/Germplasm
- Economics and Policy
- Plant breeding and Genetics
- Environmental Issues
- Alternative Fuel
- Biotechnology
- Postharvest Handling
- Seed technology

Subscribe Now!

YES!

☐ I would like to start my subscription of the **Philippine Journal of Crop Science** for year (**201**_) – __ volume: three issues
(Php 4,300 if within the Philippines or USD 120 if abroad)

☐ I would like to subscribe an issue of the journal (Php1500):
___volume ___number ___month ___year

Subscriber Details

Name

Position/Nature of work

Mailing address

Postal code/Zip Country

Website

Phone Email

☐ Cheque enclosed made payable to **Crop Science Society of the Philippines**

CROP SCIENCE SOCIETY OF THE PHILIPPINES

Officers and Board Members 2016 - 2017

Arthur R. Baria (Nestle Inc.)
President

Marissa V. Romero, PhD (PhilRice)
Vice-President

Reneeliza Jean A. Melgar (IRRI)
Secretary

Victoria C. Lapitan, PhD (PhilRice)
Treasurer

Alma O. Canama (IPB)
Auditor

Rowena H. Oane (IRRI)
Public Relations Officer

BOARD MEMBERS

Edna A. Anit, PhD (PCAARRD)

Nenita V. Desamero, PhD (PhilRice)

Tonette P. Laude, PhD (UPLB)

Apolonio M. Ocampo, PhD (UPLB)

Ma. Rebecca C. Laza, PhD (IRRI)
Ex-Officio

CSSP: A DECLARATION OF FAITH

The Crop Science Society of the Philippines is organized to promote human welfare through the discovery and dissemination of knowledge concerning the nature, utilization, improvement and interrelationships of plants and their environment, and not the least of all, the people. The society seeks to: 1) promote basic and applied research on crops and related sciences; 2) foster high quality education in crop science; 3) encourage professional advancement among it's members; 4) make current information available to workers in related fields and to the public; and 5) cooperate with other organizations in the solution of problems encountered in crop production. Thus, the Society holds a yearly scientific conference and publishes The PHILIPPINE JOURNAL OF CROP SCIENCE. All persons actively interested in any field of crop science are eligible for membership. Applications for membership should be addressed to the Treasurer of the Society and may be made on forms obtainable from her. Membership fee is PhP100 and the annual due is PhP200 per year for individual members.

Organizations interested in the objectives and programs of the Society are eligible for Sustaining Life Membership with a fee of PhP2,000 each organization. For more information, please write to The President of the Society. Address all communications to: The Crop Science Society of the Philippines, PO Box 165, College, Laguna 4031, Philippines or at pjcrops@gmail.com.

PHILIPPINE JOURNAL OF CROP SCIENCE
P.O. Box 165
College, Laguna 4031
Philippines

PRINTED MATTER
Published by the Crop Science Society of the Philippines and the Federetion of Crop Science Societies of the Philippines

Entered as Third Class Mail at the Post Office at College, Laguna, Philippines in June 2016.