INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 9, No. 3, p. 1-11, 2016

RESEARCH PAPER OPEN ACCESS

Role of biological agents and cocoa clones to control vascular streak dieback disease (*Ceratobasidium theobromae* Tallbot and Keane) of cocoa plants

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Key words: Cocoa clone, Biological agent, VSD, *Ceratobasidium theobromae*.

http://dx.doi.org/10.12692/ijb/9.3.1-11 Article published on September 17, 2016

Abstract

Cocoa (*Theobromae cacao* L) is a high economic value plantation crop and becomes one of the world's most important commodities. Indonesia is the third largest cocoa producers in the world, although the national cocoa productivity is still low, one of the causes is infectious diseases. Vascular streak dieback (VSD) disease caused by *Ceratobasidium theobromae* Tallbot and Keane has infected more than 50% cocoa cropping and caused yield losses for up to 20-30%. The purpose of this study was to test the effectiveness of the biological agent *Trichoderma asperellum*, *rhizobakteria* and *Mycorrhiza sp*. and cocoa clones to control VSD disease on cocoa crop in a screen house. The study was arranged in a split plot design with the main plot of cocoa clones consisted of three levels; while the subplot was biological agents consisted of ten levels. The research results showed that there was interaction between Sulawesi 1 clone and a combination of *Trichoderma asperellum* + *Rhizobakteria* + *Mycorrhiza* sp. inoculated with *C. theobromae*. This interaction increased as cocoa plant height (32.68 cm). Sulawesi 1 clone had the largest increases in leaf area and stem diameter, by 90.24 cm2 and 0.788 cm, respectively. Treatment combination between Sulawesi 1 clone and biological agent *Rhizobacteria* + *Mikoriza sp*. inoculated with *C. theobromae* and treatment combination between Sulawesi 7 clone and biological agent *Mikroriza sp*. inoculated with *C. theobromae* were able to decrease VSD disease incident to 33.33%.

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Introduction

As an important economic commodity, cocoa attracted several countries to increase their production. Ivory Coast supplies the world's cocoa needs by 38 % with a production of 1.5 million tons per year, while Ghana supplies around 19 % (ICCO, 2013). Meanwhile, Indonesia was ranked third with a contribution of around 13 %. Some other countries such as Nigeria, Brazil and Cameroon supply about 5 %, Equador 4 %, Malaysia 1 %, and other countries supplying 9 % (Ibiremo *et al*., 2014 ; Binam *et al*., 2008).

Indonesia's potency to increase its contribution in the global cocoa supply is still very promosing, given that some cocoa-producing provinces are still potential for improved productivity. Data from the Department of Plantation of Southeast Sulawesi (2013) showed that the acreage of cocoa reached 250.338 ha with a production of 148.746 tonnes and productivity of 821.1 kg per hectare, lower than the national productivity of 854 kg hectere, and even lower when compared with Malaysia's cocoa productivity, which reached 1,800 kg per hectare (Dormon *et al*., 2004).

One of the most important diseases in cocoa is vascular streak dieback (VSD) caused by the fungus *Ceratobasidium theobromae* Tallbot and Keane. McMahon *et al.* (2010) reported that VSD has infected cacao plants since 1980 in District Ladongi, Kolaka, and then outbrook again in Kolaka in 1989 (Askindo, 2008). VSD disease symptoms are shown by the typical symptoms of yellowing, which is causing necrosis of leaves and eventually leaves will fall. Another typical symptom is the presence of three dots on leaf petioles (Purwantara *et al*., 2009; Samuels *et al*., 2012). The fungus spreads internally to other branches, twigs or stems that can cause the death of the cocoa plant (Guest and Keane, 2007).

Despite the lack of efficient methods available to control VSD disease, it is predicted that the use of biological agents and/or resistant clones provide a good hope. This is due to biological agents have several mechanisms to control pathogens such as 1) antibiosis, which secretes chemical compounds that can kill disease-causing pathogens, 2) hyperparasit, the antagonist parasites disease-causing pathogens, and 3) competition, the competition of food or a place to live between the antagonist and disease-causing agents (Baker and Cook 1974; Keel, 2003).

It has been reported also that the use of *Rhizobacteria* and *Trichoderm* sp. reduced the incidence of fungal diseases of white root (*Rigidoporus* sp) on cashew and pepper crops (Taufik *et al*., 2011; Taufik *et al*., 2012) and tomato (Nitu *et al*., 2016). Similar results proven by Rosmana *et al.* (2014 and 2015) that the application of biological agent *Trichoderma asperellum* isolate ART-4 can significantly suppress VSD disease progression on seedlings and side-graftings.

In addition to biological agents, disease-resistant clones of VSD such as Sulawesi 1 and Sulawesi 2 have been recommended by the Indonesian's Coffee and Cocoa Research Center (Susilo and Anitasari, 2014). Susilo *et al.* (2009); and Halimah and Sri-Sukamto (2007) have proved that the Sulawesi 1 clone gave a high yield in the field, and it belongs to 11 resistant clones to xylem disease. Panda and Kush (1995) argued that the use of resistant planting material is effective in controlling a variety of pests and diseases. Therefore, this study aimed to test the effectiveness of the biological agents *Trichoderma asperellum*, *Rhizobacteria* and *Mycorrhiza* sp. and resistant clones to control VSD disease on cocoa trees in a screen house.

Materials and methods

Experimental design

This research was conducted in a screen house of the Faculty of Agriculture Halu Oleo University Kendari, conducted from January to July 2015. This study used a split plot design with the main plot was cocoa clones consisting of Sulawesi 1 clone (K1), Sulawesi 2 clone (K2) and Sultra 7 clone (K3). The subplots were biological agents, with 7 levels: without treatments and without inoculation of the fungus *C. theobromae*

(A0), without biological agents treatment and with inoculation of *C. theobromae* (A1); *Trichoderma asperellum* treatment with inoculation of *C. theobromae* (A2); rhizobacteria (*Bacillus subtilis* ST21e) treatment with inoculation of *C. theobromae* (A3); *Mycorrhiza* sp. treatment with inoculation of *C. theobromae* (A4); *Trichoderma asperellum* + rhizobacteria (*Bacillus subtilis* ST21e) treatment with inoculation of *C. theobromae* (A5); rhizobacteria (*Bacillus subtilis* ST21e) + *Mycorrhiza* sp. treatment with inoculation of *C. theobromae* (A6); *Trichoderma asperellum* + *Mycorrhiza* sp treatment with inoculation of *C. theobromae* (A7); *Trichoderma asperellum* + rhizobakteria (*Bacillus subtilis* ST21e) + *Mycorrhiza* sp. treatment with inoculation of *C. theobromae* (A8); and fungicide active ingredient Mankozob with inoculation of *C. theobromae* (A9). Therefore, there were 30 treatment combinations, and each treatment was repeated 3 times, so there were 90 treatment units. Each unit consisted of 4 plants, so overall there were 360 plants.

Preparation of planting medium and cocoa seed

Planting medium used was a mixture of soil (top soil) : goat manure : sand) with a ratio of 2 : 1 : 1. Planting medium was sterilized for 2 hours, and then put into a polybag sized 30 × 25 cm. Each polybags filled with 2 kg of the planting medium. The mucus of cocoa beans was cleaned and soaked using atonik solution for 24 hours before sowing.

Nursery and seed planting

Seedbed was done by using a container sized of 35 × 25 cm. Seedling medium was using a mixture of soil, goat manure and sand in the ratio of 2 : 1 : 1. Seedlings aged 24 days after sowing were transferred to polybags containing sterilized medium as above.

Application of biological agents and fungicide

Rhizobacteria isolate used was *Bacillus subtillis* ST21e. Rhizobacteria was applied during transplanting with a suspension dose of 10 ml per polybag. *Trichoderma asperellum* was applied through the roots during transplanting with a suspension dose of 10 ml per polybag. Application of *Mycorrhiza* sp. was done by putting 10 g *Mycorrhiza* sp. in each planting hole during transplanting. Fungicide used was fungicide with active ingredient Mankozob. Application of fungicide on crops was done by flushing a fungicide solution with a concentration of 2 ml per ml water around plant roots.

Preparation of VSD inoculum

Pathogenic fungus obtained from VSD infected leaves of cocoa plants from UPTD Field Laboratory of Crops and Horticulture Department of Southeast Sulawesi Province, in the Puuwatu District, Kendari, which was an endemic area for VSD disease. Cocoa petioles infected with fungus *C. theobromae* were cut, then soaked in 70% alcohol for 3 minutes, then put them back into a solution of 5.25 % NaOCl for 5 minutes. The petioles were again transferred into a 70% alcohol for 2 minutes, then rinsed 3 times with distilled water and dried on the filter paper.

The leaf stalks were then grown on Water Agar (WA) medium, and incubated for 3 days to obtain pathogen mycelium. After incubation, *C. theobromae* hyphae were observed microscopically in the laboratory, and then printed with the core bore.

VSD inoculation technique on cocoa plant

Inoculation of pathogens was applied on cocoa leaves aged 4 weeks after planting. *C. theobromae* mycelium which had been grown on water agar medium, was taken as many as three core bore mold, 3 mm in diameter and placed on fully open cocoa leaves with mycelium position in direct contact with the surface of the cocoa leaves. To keep the mycelium moist, water spraying was carried out regularly using a hand sprayer 2 times a day.

Cocoa plant maintenance

Plant maintenances included watering, weeding, fertilizing and pest control. Watering was done twice a day in the morning and afternoon. Weeding was done every time as necessary. Fertilization was done by making a hole around the plant. Pest control was done physically whenever the pests found.

Observed variables and data analysis

The measured variables included the increases of plant height at 8 weeks after inoculation (WAI), leaf area (cm^2) at 8 WAI, stem diameter (cm) at 8 WAI and disease incidence. VSD disease incidence (%) was carried out by observing the external symptoms of VSD on cocoa crops. Disease incidence rate was calculated using the formula:

$$KP = \frac{n}{N} \times 100\%$$

Notes:
KP = disease incidence (%)
n = number of leaves with VSD symptoms
N = total leaves observed

Data were analyzed by analysis of variance (ANOVA) using the 'R ' statistic program. The average values of treatments were compared using Duncan Multiple Range Test (DMRT) at α = 0.05.

Results and discussion

Plant height

There was interaction between the applications of biological agents and cocoa clones on plant height. Interaction occurred on treatment combination of Sulawesi 1 clone with biological agent *Trichoderma asperellum* + *Rhizobacteria* + *Mycorrhiza* sp. inoculated with *C. theobromae* (K1A8) resulted in 32.68 cm of plant height, and was significantly different with the treatment of Sulawesi 2 clone with a biological agent *Trichoderma asperellum* + *Rhizobacteria* + *Mycorrhiza* sp. inoculated with *C. theobromae* (K2A8) (25.97 cm) (Table 1).

Plant height resulted from the treatment of Sulawesi 1 clone with Rhizobacteri inoculated with *C. theobromae* (K1A3) was also significantly different with treatment of biological agent rhizobacteris and Sultra 7 clone inoculated with *C. theobromae* (K3A3) (Table 1).

Table 1. Interaction between cocoa clones and biological agents on cocoa plant height (cm), 8 weeks after inoculation with fungus *C. theobromae*.

Biological agents	Sulawesi 1 clone (K1)	Sulawesi 2 clone (K2)	Sultra 7 clone (K3)
Without treatments and without inoculation with fungus *C. theobromae* (A0)	24.87^{STU} a	23.72^{QR} a	25.31^{PQR} a
Without biological agents treatment and with inoculation of *C. theobromae* (A1)	22.46^{U} a	20.52^{R} a	20.89^{R} a
Trichoderma asperellum treatment with inoculation of *C. theobromae* (A2)	29.84^{PQR} a	25.40^{PQ} a	24.13^{PQR} a
Bacillus subtilis ST21e treatment with inoculation of *C. theobromae* (A3)	31.25^{PQ} a	25.05^{PQR} ab	22.41^{QR} b
Mycorrhiza sp. treatment with inoculation of *C. theobromae* (A4)	24.79^{STU} a	27.81^{PQ} a	24.92^{PQR} a
Trichoderma asperellum + *Bacillus subtilis* ST21e treatment with inoculation of *C. theobromae* (A5)	29.06^{PQRS} a	24.20^{QR} a	25.51^{PQR} a
Bacillus subtilis ST21e) + *Mycorrhiza* sp. treatment with inoculation of *C. theobromae* (A6)	27.56^{QRST} a	29.54^{P} a	26.17^{PQ} a
Trichoderma asperellum + *Mycorrhiza* sp treatment with inoculation of *C. theobromae* (A7)	25.24^{RSTU} a	28.47^{PQ} a	27.18^{PQ} a
Trichoderma asperellum + *Bacillus subtilis* ST21e + *Mycorrhiza* sp. treatment with inoculation of *C. theobromae* (A8)	32.68^{P} a	25.97^{PQ} b	28.35^{P} ab
Fungicide with inoculation of *C. theobromae* (A9).	23.72^{TU} a	26.02^{PQ} a	25.68^{PQR} a

Note: The values followed by the same letters in the same column (PQRSTU) or rows (abc) are not significant by DMRT at α 0.05.

An increase in plant height allegedly because the use of rhizobacteria, alone or in combination with *T. asperellum* + *Mycorrhiza* sp., was able to provide nutrients and hormones, that stimulated plant growth. Research results by Taufik *et al.* (2011) revealled that cashew crop which had been given a biological agent produced cashew plant height 11.58 cm higher than the control. Swain *et al.* (2007) stated that yams (*Dioscorea rotundata*) inoculated with IAA-producing bacteria *Bacillus subtilis* significantly had increased roots, stem ratio, higher than non inoculated plants. IAA mediates the production of ethylene which roles in increasing root biomass, number of hair roots and surface area of tomato roots that were inoculated with plant growth promoting rhizobacteria (PGPR) (Ribaudo *et al.*, 2006).

According to Khaeruni *et al.* (2011), isolates of *Bacillus subtilis* ST21e had the ability to produce indole acetic acid (IAA) amounted to 59.44 ppm and had the ability as phosphate solvent and nitrogen fixing nonsimbiotic.

In addition, rhizobacteria can also mobilize the absorption of various nutrients in the soil as well as synthesizing and changing the concentration of a variety of phytohormones (Park *et al.*, 2009; Bhattacharyya and Jha, 2012).

Tabel 2. The average increase in leaf area and stem diameter by the treatments of biological agents and clones, 8 weeks after inoculation with fungus *C. theobromae*.

Treatment	Leaf area (cm^2)	Stem diameter (cm)
Main plot		
Sulawesi 1 clone (K1)	90.24	0.788
Sulawesi 2 clone (K2)	78.10	0.773
Sultra 7 clone (K3)	79.91	0.730
Sub plot		
Without treatments and without inoculation with fungus *C. theobromae* (A0)	72.12^{b}	0.737^{bc}
Without biological agents treatment and with inoculation of *C. theobromae* (A1)	71.01^{b}	0.708^{c}
Trichoderma asperellum treatment with inoculation of *C. theobromae* (A2)	77.93^{ab}	0.821^{a}
Bacillus subtilis ST21e treatment with inoculation of *C. theobromae* (A3)	91.86^{ab}	0.783^{ab}
Mycorrhiza sp. treatment with inoculation of *C. theobromae* (A4)	98.57^{a}	0.804^{ab}
Trichoderma asperellum + *Bacillus subtilis* ST21e treatment with inoculation of *C. theobromae* (A5)	72.50^{b}	0.769^{abc}
Bacillus subtilis ST21e) + *Mycorrhiza* sp. treatment with inoculation of *C. theobromae* (A6)	89.84^{ab}	0.761^{abc}
Trichoderma asperellum + *Mycorrhiza* sp treatment with inoculation of *C. theobromae* (A7)	88.09^{ab}	0.752^{abc}
Trichoderma asperellum + *Bacillus subtilis* ST21e + *Mycorrhiza* sp. treatment with inoculation of *C. theobromae* (A8)	90.34^{ab}	0.754^{abc}
Fungicide with inoculation of *C. theobromae* (A9).	75.27^{b}	0.749^{bc}
CV main plot (%)	7.66	8.37
CV sub plot (%)	5.13	14.40

Note: The values followed by the same letters in the same column are not significant by DMRT at α 0.05.

The existence of *mycorrhiza* in the combined treatment of biological agents may have a role for effective absorption of nutrients required by plants. Sarmin *et al.* (2012) also proved that the use of *Mycorrhiza* sp. can encourage cashew crop growth, 14 cm higher than the control, although being inoculated with white root fungus (*Rigidoporus* sp.). Anggarani *et al.* (2015) stated that the increase in plant height of sweet sorghum (*Sorghum bicolor* L. Moench) applied with *Mycorrhiza* sp. was due the effective absorption of nutrients. In addition, the mycorrhiza allegedly role the phosphate producing bacteria to stay longer around the plant roots (Abbaspour *et al.*, 2015). Rosalina and Hilman (2005) reported that phosphate-solubilizing bacteria stay longer around the roots of *Mycorrhiza* sp. infected corn compared to plants without mycorrhiza.

Tabel 3. Interaction between cocoa clones and biological agents on VSD disease incidence (%) on cocoa plants, 8 weeks after inoculation.

Average disease incident	Sulawesi 1 clone (K1)	Sulawesi 2 clone (K2)	Sultra 7 clone (K3)
Without treatments and without inoculation with fungus *C. theobromae* (A0)	0.00 S a	0.00 R a	0.00 T a
Without biological agents treatment and with inoculation of *C. theobromae* (A1)	83.33 PQ a	100.00 P a	100.00 P a
Trichoderma asperellum treatment with inoculation of *C. theobromae* (A2)	66.67 PQR a	66.67 Q a	75.00 QR a
Bacillus subtilis ST21e treatment with inoculation of *C. theobromae* (A3)	91.67 P a	58.33 Q b	66.67 QRS b
Mycorrhiza sp. treatment with inoculation of *C. theobromae* (A4)	66.67 PQ a	50.00 Q ab	33.33 S b
Trichoderma asperellum + *Bacillus subtilis* ST21e treatment with inoculation of *C. theobromae* (A5)	75.00 PQ a	66.67 Q a	83.33 PQ a
Bacillus subtilis ST21e) + *Mycorrhiza* sp. treatment with inoculation of *C. theobromae* (A6)	33.33 R a	58.33 Q a	50.00 RS a
Trichoderma asperellum + *Mycorrhiza* sp treatment with inoculation of *C. theobromae* (A7)	83.33 PQ a	75.00 Q ab	41.67 RS b
Trichoderma asperellum + *Bacillus subtilis* ST21e + *Mycorrhiza* sp. treatment with inoculation of *C. theobromae* (A8)	58.33 QR a	66.67 Q a	66.67 QRS a
Fungicide with inoculation of *C. theobromae* (A9).	91.67 P a	75.00 Q a	66.67 QRS a

Note: The values followed by the same letters in the same column (PQRST) or rows (abc) are not significant by DMRT at α 0.05.

The involvement of *Trichoderma* sp. in treatment combinations allegedly contributed as a complement in providing plant nutrients, especially phosphorus nutrient (Hardiatma, 2008). In addition to the role of providing nutrients, *Trichoderma* sp. also played a role in helping to establish ectomycorrhiza. This is in line with the results of the research conducted by Widyastuti (2007) that the percentage of ectomycorrhizal infection in roots increased with the addition of *Trichoderma* sp.

Leaf Size

Although not significantly different, Sulawesi 1 clone (K1) had the highest average of leaf area (90.24 cm^2), and then consequtively followed by Sultra 7 clone (K3) by 79.91 cm^2 and Sulawesi 2 clone (K2) by 78.10

cm². Meanwhile, independently, the application of biological agents *Mycorrhiza* sp. was able to accelerate cocoa leaf area growth (up to 98.57 cm²) although it had been inoculated with *C. theobromae*., and significantly different from the treatment of without biological agents with inoculation of *C. theobromae* (A1), A0, A5, and A9 (Table 2). This was presumably because *Mycorrhiza* sp.was able to absorb the nutrients in the soil and then tranported to the plant through the roots. In addition, *Mycorrhiza* sp. had a phosphatase enzyme that can hydrolyze phosphate bonded with Fe and can also produce hormones such as auxin, gibberellins and cytokinins to stimulate the growth of plants (Hardiatma, 2008).

Stem diameter

There was no significant difference in diameter of cocoa plants for each clone tested, yet Sulawesi 1 clone (K1) had a diameter average of 0.788 cm, while Sulawesi 2 clone (K2) 0.773 cm and Sultra 7 clone (K3) 0.730 cm. However, the application of *Trichoderma asperellum* was able to stimulate the growth of cocoa stem diameter (0.821 cm) although it had been inoculated with *C. theobromae*, and significantly different from the biological agents-untreated clone with inoculation of *C. theobromae* (A1), without biological agent treatment and without *C. theobromae* fungus inoculation (A0), and fungicide treatment with inoculation of *C. theobromae* (A9) (Table 2). This was presumably due to the ability of *Trichoderma asperellum* to provide sufficient nutrients for plant, especially phosphor. The same findings reported by Aulia *et al.* (2014) that the increase in diameter of copper rod "meranti" (*Shorea leprosula* Miq) in nursery triggered by phosphorus nutrient availability produced by *Trichoderma* sp.

Disease incidence of vascular streak dieback (C. theobromae) on cocoa plant

Interactions occurred between Sulawesi 1 clone and combination treatments of *Rhizobacteria* + *Mycorrhiza* sp., with inoculation of *C. theobromae* (K1A6), but no significantly different with other treatments. Similarly, there was interaction between Sultra 7 clone treatment and treatment of biological agent *Mycorrhiza* sp. inoculated with *C. theobromae* (K3A4). Both interactions provided the lowest disease incidence (33.33%) (Table 3).

The low incidence of VSD disease on the application of biological agents combination treatment of *Rhizobacteria* + *Mycorrhiza* sp. with inoculation of *C. theobromae* (A6) allegedly due the capability of *Rhizobacteria* to directly produce secondary metabolites such as siderophores, HCN, enzymes, extracellular and antibiotic compounds, or indirectly induce plant resistance to pathogen infection (Kazempour, 2004; Kim *et al*., 2008). The indicators of the induction of plant resistance against pathogen infection caused by the treatment of rhizobacteria had previously been reported such as, among others, an increase in activity of peroxidase, phenylalanine ammonia-lyase, polyphenol oxidase and the production of phytoalexin compounds (Silva *et al*., 2004; Hoerussalam *et al*., 2013).

Bacillus subtilis ST21e used in this study had been proved by Khaeruni *et al.* (2011) were able to suppress the development of fusarium wilt in tomatoes and wilted Rhizoctonia in soybeans, and can stimulate vegetative growth of the plants.

Research results also showed that the use *Mycorrhiza* sp., independently or in combination with *Rizobacteria* was able to suppress *C. theobromae* fungal infections. This is in line with research results of Noveriza *et al.* (2005) that *Mycorrhiza* sp. combined with biological agents can suppress *Phytophthora capsici* disease on pepper plant nursery. Similarly, the use of *Mycorrhiza* sp. independently on soybean crop can control *Streptomyces rolfsii* by 75 % (Setiawan *et al*., 2014). *Mycorrhiza* sp. treatment on plants stimulated plant growth. This was also revealed by Mosse (1981), that the addition *Mycorrhiza* sp. changed the physiology of host plants, where growth and resistance to environmental stress increased. According to Setiawan *et al.* (2014), the use of *Mycorrhiza* sp. suppressed the incidence of disease, also reduced the need for chemical fertilizers by 25% and increased the

production of soybeans.

Conclusion

The interactions occurr between Sulawesi 1 clone (K1) and the combined treatment of biological agents *Trichoderma asperellum* + *Rhizobacteria* + *Mycorrhiza* sp. inoculated with *C. theobromae* (K1A8) in stimulating the increase of plant height (32.68 cm). Sulawesi 1 clone has a highest response of leaf area and stem diameter by 90.24 cm^2, and 0.788 cm, respectively. Combined treatments of Sulawesi 1 clone with the treatment of biological agents *Rhizobacteria* + *Mycorrhiza* sp. inoculated with *C. theobromae* (K1A6) and combined treatment of Sultra 7 clone with the treatment of biological agents *Mycorrhiza* sp. inoculated with *C. theobromae* (K3A4), are able to suppress the incidence of VSD disease down to 33.33 %. The use of biological agents can be recommended and gives great hope for use in VSD disease control.

Acknowledgement

This study was part of a cooperative research partnership and national agricultural development (KKP3N) financed through Research and Development Fund of the Ministry of Agriculture of the Republic of Indonesia, with Contract Number: 44.13/HM.240/1.1/3/2015, dated March 5, 2015.

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