MICROBIOLOGY INDONESIA
ISSN 1978-3477
Vol 4, No 3, Dec 2010, p 137-142

Available online at:
http://www.permi.or.id/journal/index.php/mionline
DOI: 10.5454/mi.4.3.7

Identification and Selection of Entomopathogenic Fungi as Biocontrol Agents for *Aphis gossypii* from South Sumatra

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Aphid, *Aphis gossypii* is a vector of curly virus disease. The damage of chili due to its feeding is only 35% and it can achieved 100% if the damage caused by the aphid as a vector. The objectives of this research were to explore, to isolate, to identify, and to select entomopathogenic fungi as biocontrol agents for *A. gossypii*. The fungi were explored using insect bait in soil and collected infected insects from South Sumatra, Indonesia. Then, the fungi were isolated and identified, and finally the bioefficacy tests were done using 1 x 10^6 conidia mL^{-1} against the third instar of *A. gossypii.* The explorations found 25 isolates of enthomopathogenic fungi consisting 10 isolates of *Beauveria bassiana* and 15 isolates of *Metarhizium anisopliae.* Selection of the fungi isolates on the aphid nymphs showed that isolate BPM isolated from *Pseudoplusia chalcites* caused the highest mortality rate (80.80%), while the lowest (47.20%) was caused by the isolate BAgTb isolated from *A. gossypii.* The shortest time needed to produce 50% mortality (Lethal Time $_{50}$) was 2.54 days (isolate of *Chrysodeixis chalcites from Muarasiban*). The longest time (3.66 days) was produced by isolate of *Tenebrio molitor from Tanjung Raja*.

Key words: *Aphis gosypii*, *Beauveria, Metarhizium*, fungi

The most important pest for chili is *Aphis gossypii,* which is the vector of curly virus disease Aphid, *Aphis gossypii* is a vector of curly virus disease. The damage of chili due to its feeding is only 35% and it can achieved 100% if the damage caused by the aphid as a vector (Fuller *et al*. 1999). *A. gossypii* is a carrier for 76 viruses attacking various host plants (Satar *et al*. 1999) and has been reported resistant to various insecticides (Wang *et al.* 2002).

It is crucial to identify an alternative control that is relatively safer for both agricultural product and environmental health. Biological control is the main component of integrated pest management (IPM) that is a safer pest control than other control methods (Lopes *et al*. 2009). Biological control is needed for green consumers in the world who prefered pesticide free agricultural products. This can be achieved by controlled biologically of the vector insects using natural enemies, such as entomopathogen.

Beauveria bassiana and *Metarhizium anisopliae* are common soil-borne entomopathogenic fungi that occur worldwide. *B. bassiana* cause a disease known as the white muscardine disease because infected insects covered with a layer of white mold (Alves *et al.* 2002; Klinger *et al.* 2006) and the green muscardine disease for *M. anisopliae* (Santiago *et al.* 2001). Both fungi attack the immature and adult stages of several insect orders, such as Hemiptera (Liu *et al*. 2002) and Diptera (Moraga *et al*. 2006). In order to develop successful biological control, a basic research is needed to find entomopathogenic fungi which are most pathogenic againts the vector, *A. gossypii.* Therefore, the objectives of this research were to explore, to isolate, to identify, and to select entomopathogenic fungi as biocontrol agents for *A. gossypii*.

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MATERIALS AND METHODS

Exploration of Entomopathogenic Fungi. Entomopathogenic fungi exploration was done by using two methods to obtain many species or strains of the fungi. The first method by collecting aphid nymphs and adults, larvae of Lepidoptera, Hemiptera, and other insect ordo that were sick or dead due to fungus infection (Herlinda *et al.* 2008). The infected insects that showed symptoms of dry body and the presence of conidia and fungal conidia, white or green body of the larvae were isolated or purified. Surveys to explore the fungi were carried out five times on each location. Then, the fungus-infected insects were isolated in the laboratory at a cabinet of laminar air flow that had been sterilized with 70% alcohol.

The second method of fungus exploration was to use insect as bait following method of Hashim and Azwana (2003). The insect used was third instar of *Tenebrio molitor* (hongkong caterpillar) that had been newly molting. Soil used to trap the fungi was taken by purposive sampling from forests in South Sumatra. The soil sample was taken by digging at a depth of 50-10 cm, brought to the laboratory as much as 400 g, and then it was put into a plastic tray (13 x 13 x 10 cm^3). The hongkong caterpillars were immersed 0.5 cm deep in the soil, and 20 larvae of the caterpillars were put in bottom of the tray. This treatment was repeated 20 times. Then, the tray was covered with a piece of black cloth that had been moistened. Three days later, the infected caterpillars were examined and isolated in the laboratory.

Isolation and Identification of Entomopathogenic Fungi. Isolation of entomopathogenic fungi used methods of Herlinda *et al.* (2006). The fungus-infected insects and caterpillars were sterilized with 1% sodium hypochlorite or 70% alcohol for three minutes. Then insects were rinsed with sterile water three times, and dried on top of sterile filter paper. Then, they were placed in a petri dish (diameter 9 cm) containing moist sterile paper and incubated to stimulate

conidial germination. Fungi were isolated, cultured on Saborroud Dextrose Agar (SDA) medium, and incubated for seven days at 25-27°C and relative humidity 80-85%. Then, a pure culture fungus was identified by using reference of Toledo *et al.* (2010).

The isolate was grown on slants of SDA medium supplemented with chitin from the small mole cricket, kept in 1.5 x 13 cm glass test tubes, and incubated for 7 days. The fungal spores were harvested from the slant culture, and each 1 g medium of the isolate was separately suspended in 9 mL water. Conidial density was calculated after analysis of a 1 mL sample of the suspension in a haemocytometer.

Conidial germination as a measure of viability was obtained by spreading $1x10^6$ propagules in 100 µL on glucose yeast agar (GYA) medium. The suspension was incubated at room temperature for 24 h. To calculate conidial viability per unit volume, total counts estimated with the haemacytometer were multiplied by the percentage of germination.

Selection of Entomopathogenic Fungi as Biocontrol Agents. After culturing isolates of the fungi, *A. gossypii* was also cultured on the chili to get the available colony that would be used to entomopathogenic fungi selection. The selection was done using method of Herlinda *et al.* (2008). Conidia of the fungal isolates were used by dripping topically 10 µL fungal suspension (density of $1x10^6$ conidia mL^{-1}) on the third instar of *A. Gossypii*. In this experiment, each isolate (Table 1) was inoculated on 25 newly molting-third instar of *A. gossypii* and repeated five times. Nymphs that had been exposed to the fungus conidia were subsequently maintained in plastic cylinders (diameter 9 cm and height 30 cm) covered with cloth. In the cylinders, there was a pot (diameter 9 cm) of chili growth. Every 6 h during the nymph stage, the number of dead nymph were recorded, while the number of nymphs growing to be adults were also recorded daily until all the nymphs became adult.

Data Analysis. The difference of mortality data and adult percent emerging were analyzed using analysis of variance, with the Tukey test. Time of nymph death was analyzed to determine the LT_{50} using probit analysis and also calculated by using SAS-STAT program.

RESULTS

Isolates of Entomopathogenic Fungi. Two species of entomopathogenic fungi from South Sumatra were identified, *B. bassiana* and *M. anisopliae. B. bassiana* consisted of 10 isolates, whereas *M. anisopliae* were 15 isolates (Table 1). The fungi could be found in lowland and highland areas of South Sumatra, however they were more found in lowland areas than in highlands. Exploration method by dipping bait insecs in the soil was more effective than collecting infected insects from the field. *M. anisopliae* isolates more often found during survey than *B. bassiana* isolates.

Table 1 Entomopathogenic fungi isolates collected from South Sumatra

Isolate codes	Source host insects	Origins	Exploration methods
Lowland areas			
BAgTb	*Aphis gossypii*	Talang Buruk	Collection of infected insects
BNlPTr	*Nilaparvata lugens*	Pantura	Collection of infected insects
BTmSo	*Tenebrio molitor*	Soak	Bait insects in soil
BTmTb	*Tenebrio molitor*	Talang Buruk	Bait insects in soil
MaAgIn	*Aphis gossypii*	Inderalaya	Collection of infected insects
MaLaIn	*Leptocorisa acuta*	Inderalaya	Collection of infected insects
MAgIn	*Aphis gossypii*	Inderalaya	Collection of infected insects
MLaPTr	*Leptocorisa acuta*	Pantura	Collection of infected insects
MTmBb	*Tenebrio molitor*	Bukit besar	Bait insects in soil
MTmIn	*Tenebrio molitor*	Inderalaya	Bait insects in soil
MTmGb	*Tenebrio molitor*	Gelumbang	Bait insects in soil
MTmKt	*Tenebrio molitor*	Kenten	Bait insects in soil
MTmTk	*Tenebrio molitor*	Talang Kelapa	Bait insects in soil
MTmTr	*Tenebrio molitor*	Tanjung Raja	Bait insects in soil
Highland areas			
BLePd	*Lipaphis erysimi*	Pagardin	Collection of infected insects
BPM	*Chrysodeixis chalcites*	Muarasiban	Collection of infected insects
$BPcPd_1$	*Chrysodeixis chalcites*	Pagardin	Collection of infected insects
$BPcPd_2$	*Chrysodeixis chalcites*	Pagardin	Collection of infected insects
BTmPd	*Tenebrio molitor*	Pagardin	Bait insects in soil
BCcC	*Chrysodeixis chalcites*	Curup	Collection of infected insects
MAgPd	*Aphis gossypii*	Pagardin	Collection of infected insects
MTmBk	*Tenebrio molitor*	Bedeng Kresek	Bait insects in soil
MTmJr	*Tenebrio molitor*	Jarai	Bait insects in soil
MTmKj	*Tenebrio molitor*	Kerinjing	Bait insects in soil
MTmMs	*Tenebrio molitor*	Muarasiban	Bait insects in soil

Isolate code using first letter B refers to *Beauveria bassiana* and M refers to *Metarhizium anisopliae.*

Table 2. Conidial density of *Beauveria bassiana* and M*etarhizium anisopliae* isolates

Isolate codes	Conidial density (x 10^6 conidia mL^{-1})	
	Range	Mean±SE
Beauveria bassiana		
BAgTb	36.60-37.40	37.00 ±0.40 ef
BCcC	21.55-22.23	21.87 ±0.34 abc
BLePd	43.30-45.15	43.98 ±1.02 f
BNlPTr	20.60-43.35	21.53 ±0.54 abc
BPM	37.15-59.60	45.39 ±12.36 f
$BPcPd_1$	24.60 -27.52	26.25 ±1.49 cd
$BPcPd_2$	20.60-22.83	21.61 ±1.13 abc
BTmPd	42.17-43.65	42.73±0.80 f
BTmSo	17.23-21.23	19.85±2.27 ab
BTmTb	20.40 -24.52	22.81±2.15 abc
Metarhizium anisopliae		
MaAgIn	19.08-20.25	19.63±0.59 a
MAgIn	37.40-38.00	37.78±0.33 ef
MAgPd	43.95-44.85	44.46±0.46 f
MaLaIn	19.37-19.45	19.38±0.04 a
MLaPtr	17.88-20.90	19.74±1.63 a
MTmBb	18.75-20.45	19.36±0.95 a
MTmBk	38.05-39.20	38.72±0.59 ef
MTmGb	29.50-34.37	31.98±2.44 f
MTmIn	35.70-38.47	38.96±3.53 ef
MTmJr	36.87-38.70	37.62±0.96 ef
MTmKj	42.45-44.70	44.06±1.40 f
MTmKt	27.65-23.35	25.05±0.32 bc
MTmMs	42.87-43.42	43.10±0.29 f
MTmTk	18.00-18.63	18.36±2.29 a
MTmTr	38.37-44.27	41.98±3.16 f

Data in the same column followed by the same letter showed they were not significantly different (HSD test, P 0.05).

Table 3. Conidial viability of *Beauveria bassiana* and M*etarhizium anisopliae* isolates

Isolate codes	Percentage of conidial viability (%)	
	Range	Mean±SE
Beauveria bassiana		
BAgTb	14.30-24.20	20.67±5.52 abcd
BCcC	19.89-33.29	25.20±7.12 abcdef
BLePd	20.00-28.10	25.20±4.51 abcdef
BNlPTr	14.80-20.30	16.80±3.04 abc
BPM	38.10-47.50	41.87±4.97 f
$BPcPd_1$	22.72-34.78	28.37±6.07 abcdef
$BPcPd_2$	14.83-31.37	21.50±8.72 abcde
BTmPd	12.50-19.40	16.01±3.45 abc
BTmSo	29.03-36.40	36.58±3.79 def
BTmTb	26.30-40.90	31.5 ±8.16 bcdef
Metarhizium anisopliae		
MaAgIn	5.49-21.37	13.15±7.95 ab
MAgIn	10.50-15.15	13.15±2.39 ab
MAgPd	15.40-23.50	18.83±4.19 abcd
MaLaIn	9.69-18.28	14.80±4.52 abc
MLaPtr	15.70-21.53	16.80±2.92 abcd
MTmBb	8.71-14.47	11.9±2.93 a
MTmBk	10.80-17.80	14.87±3.64 abc
MTmGb	25.00-29.80	28.13±2.72 abcdef
MTmIn	20.70-34.60	26.09±7.46 abcdef
MTmJr	14.30-24.20	20.67±5.52 abcd
MTmKj	20.00-27.02	23.61±3.51 abcdef
MTmKt	28.00-43.50	32.21±7.89 cdef
MTmMs	20.00-27.60	21.00±6.16 abcd
MTmTk	15.68-26.71	21.70±5.58 abcde
MTmTr	31.43-53.30	39.91±11.7 ef

Data in the same column followed by the same letter showed they were not significantly different (HSD test, P 0.05).

Conidial Density and Viability of Entomopathogenic Fungi. *B. bassiana* and *M. anisopliae* isolates had very dense conidia, but the trend of conidial density of *B. bassiana* isolates was significantly higher compared to *M. anisopliae* isolates (Table 2). The highest conidial density of entomopathogenic fungi reached 45.39x10^6 conidia mL^{-1} that was found on *B. bassiana* isolates coded BPcM, the lowest found on MTmTk isolate of *M. anisopliae* (18.36x10^6 conidia mL^{-1}).

The highest conidial viability of entomopathogenic fungi reached 47.50% with a 41.87% average viability that was found in *B. bassiana* isolates coded by BPcM (Table 3). The lowest conidial viability averaged of 11.90% found on *M. anisopliae* isolates coded by MTmBb. The conidial viability of *B. bassiana* isolates tended significantly higher than those of *M. anisopliae* isolates.

Virulence Isolates of Entomopathogenic Fungi. Twenty five isolates were selected to determine the most virulent isolate *againts A. gossypii* nymphs. The results showed that all *B. bassiana* and *M. anisopliae* isolates were pathogenic to the nymphs of *A. gossypii* causing mortalities between 42.40 and 80.80% (Table 4). The most virulent isolate was BPM of *B. bassiana* isolate causing an average of 80.80% mortality, and the two least ones were BAgTb of *B. bassiana* isolate and MLaPtr of *M. anisopliae* isolate. *A. gossypii* mortality caused by BPM isolate was significantly different from BAgTb and MLaPtr isolates. BPcM isolate was isolated from *Chrysodeixis chalcites* in highland areas, BAgTb isolate from *Aphis gossypii*, and MLaPtr isolate from *Leptocorisa acuta.* Both of the BAgTb *and* MLaPtr isolates were from lowland areas. The level of *A. gossypii* mortality caused by *B. bassiana* treatment tended to be higher than those of *M. anisopliae* treatment.

All *B. bassiana* and *M. anisopliae* isolates were able to infect *A. gossypii* nymphs and almost had low value of lethal time median (Lt_{50}). The time median when death occured differed among isolates and it varied between 2.54 and 3.66 days. (Table 5). The result indicated that BPM isolate of *B. bassiana* had the lowest LT_{50} value (2.54 days) againts *A. gossypii* nymphs, while MTmTr isolate of *M. anisopliae* was the highest one (3.66 days).

DISCUSSION

In this study, exploration methods that were able to find entomopathogenic fungi were deeping insect bait in the soil and collecting the infected insects from the fields. Preliminary survey following method of Feng *et al.* (2007) had tried to find the fungal conidia of entomopathogen from air using sticky cards but they were unable to be found. The fungi were easier to be found from the soil compared to infected insects from the fields. Herlinda *et al*. (2008) found that the entomopathogenic fungi obtained from infected

Table 4 Mortality of *Aphis gossypii* nymph exposed to conidia of *Beauveria bassiana* and *Metarhizium anisopliae* at a concentration of 10^6 conidia mL^{-1}

Isolate codes	Nymph mortality (%)	
	Range	Mean ± SE
Beauveria bassiana		
BAgTb	32-60	42.40±13.44 a
BCcC	36-64	47.20±12.13 ab
BLePd	24-72	52.80±17.29 ab
BNlPTr	32-64	46.40±13.44 ab
BPM	72-92	80.80± 7.69 b
BPcPd	52-84	67.20±14.25 ab
BPcPd2	32-64	47.20±13.38 ab
BTmPd	36-52	44.80± 7.15 ab
BTmSo	32-76	53.60±21.46 ab
BTmTb	52-80	54.40±16.63 ab
Metarhizium anisopliae		
MaAgIn	44-60	53.60± 6.69 ab
MagIn	20-92	47.20±29.03 ab
MagPd	32-68	48.00±16.73 ab
MaLaIn	36-68	49.60±13.73 ab
MLaPtr	32-64	42.40±17.57 a
MTmBb	32-52	44.80± 7.69 ab
MTmBk	52-60	53.60± 8.29 ab
MTmGb	40-80	54.40 ±15.3 ab
MtmIn	36-64	47.20±11.09 ab
MTmJr	40-80	57.60±10.43 ab
MTmKj	28-88	46.40±23.93 ab
MTmKt	40-80	56.80±14.80 ab
MTmMs	32-68	52.00±12.96 ab
MTmTk	36-68	50.40±15.12 ab
MTmTr	36-56	44.00± 7.48 ab

Data in the same column followed by the same letter showed they were not significantly different (HSD test, P 0.05).

insects tended to be more difficult to be isolated. The other from the infected insects often were contaminated by air fungi. Hashim and Azwana (2003) reported that the conidia in the soil tended to be more persistent, they could be easily trapped using insect bait. Fuxa and Richter (2004) found that soils with high clay content improved persistence of the fungal conidia. Thus, future research using clay will be required to formulate the fungus conidia to increase efficacy as biological control.

Condial density observed were densed, and the highest conidial density of entomopathogenic fungi reached $45.39x10^6$ conidia mL^{-1} that was found on *B. bassiana* isolates coded by BPcM. Soundarapandian and Chandra (2007) stated that conidial density were determined by mass production media and temperature of the incubated room. Liquid media tended to produce more conidia than those of solid ones. The optimum temperature and ideal pH for the mass production of *M. anisopliae* was found to be 25-30°C and 7, respectively.

Few germinated conidia were observed at 24 h of incubation that only reached 47.50%. The fungal isolates used in this study generally had low viability, it could be caused by shorter time (24 h) used in incubation of conidial suspension. Conidia were considered germinated if germtube lengths were two times in diameter of the propagules or if with conspicuous swelling (Toledo *et al*. 2010). They reported that germinated conidia in vitro for *B. bassiana* and *M. anisopliae* at 72 hours could be 95.50% and 100%, respectively. Bidochka *et al*. (2000) stated that

Table 5 Lethal time median (LT_{50}) of *Aphis gossypii* nymph exposed to conidia of *Beauveria bassiana* and *Metharizium anisopliae* at a concentration of 1×10^6 conidia mL^{-1}

Isolate codes	Mean LT_{50} (days)	95% Confidence limit		Regression Equation
		Lower	Upper	
Beauveria bassiana				
BAgTb	3.18	2.97	3.50	y= 0.176+0.066x
BCcC	3.02	2.73	3.53	y= 0.155 +0.061x
BLePd	2.91	2.69	3.23	y= 3.070+1.060x
BNlPTr	3.06	2.83	3.39	y= 0.167+ 0.065x
BPM	2.54	2.42	2.67	y= 0.194+ 0.077x
BPcPd1	2.80	2.67	2.96	y= 0.194 +0.075x
BPcPD2	3.02	2.72	3.56	y= 0.194 +0.075x
BTmPd	3.08	2.85	3.44	y= 2.980 +0.097x
BTmSo	3.10	2.86	3.46	y= 0.185 +0.078x
BTmTb	3.08	2.84	3.43	y= 0.157 +0.062x
Metarhizium anisopliae				
MaAgIn	2.81	2.56	3.21	y= 0.142 + 0.058x
MAgIn	3.03	2.78	3.42	y= 2.890 + 0.950x
MAgPd	3.02	2.75	3.47	y= 0.188 + 0.079x
MaLaIn	2.89	2.57	3.48	y= 0.144 + 0.058x
MaLaPTr	3.11	2.80	3.71	y= 0.151 + 0.060x
MTmBb	3.09	2.81	3.55	y= 0.155 + 0.062x
MTmBk	2.95	2.75	3.25	y= 0.166 + 0.060x
MTmGb	3.03	2.89	3.20	y= 3.060 + 1.010x
MTmIn	3.14	3.10	3.89	y= 0.127 + 0.047x
MTmJr	2.87	2.63	3.26	y= 3.050 + 1.060x
MTmKj	3.20	2.93	3.39	y= 0.179 + 0.069x
MTmKt	3.23	2.98	3.60	y= 0.135 + 0.049x
MTmMs	3.07	2.92	3.27	y= 0.910 + 0.073x
MTmTk	2.91	2.62	3.39	y= 2.820 + 0.970x
MTmTr	3.66	3.36	4.13	y= 0.155 + 0.540x

conidial viability was determined by temperature. The optimum temperature needed for entomopathogenic fungal conidia to germinate was 22-27°C with optimum humidity above 90%, and at under 86% humidity, the virulence would decrease continuously.

Twenty five isolates of *B. bassiana* and *M. anisopliae* found in this reasearch were almost pathogenic againts *A. gossypii* nymphs. The most virulent isolate was BPM of *B. bassiana* isolate causing an average of 80.80% mortality. BPcM isolate was isolated from *Chrysodeixis chalcites* that was unrelated to *A. gossypii.* No relationship between pathogenicity and the origin of the isolates was observed. BagTb of *B. bassiana* isolate isolated from *A. gossypii* had the lowest pathogenicity and caused only 42.40% mortality of *A. gossypii*. Liu *et al*. (2002) also found that virulent isolates of *B. bassiana* or *M. anisopliae* could be originally isolated from related and unrelated hosts. The ability of the BPcM isolate to produce the highest mortality rates might be caused either by their genetic characteristics, or by their conidial viability. Aregger (1992) stated that conidial viability factor might be the factor affecting virulence. Rate of loss of conidial viability of *B. bassiana* varies among the strain. Decline of conidial viability of this fungus correlated with decline of host mortality due to its infection.

B. bassiana and *M. anisopliae* isolates needed just 2.54 days and 2.81 days, respectively to kill *A. gossypii*. It takes shorter time than they killed other species of host, such as planthopper *Peregrinus maidis* (Toledo *et al.* 2010). Thompson and Brandenburg (2005) reported that death caused by the fungi usually occured more than 48 h after attachment of conidia to the insect cuticle. Toledo *et al.* (2010) found that germ tubes on host cuticular surface began to be found at 24 and 48 h, and they were observed penetrating directly through the host cuticle in regions near the hairs of the second antennal segment and on the laterosternites of abdomen. After 72 h, long and errant germ tubes were detected on the cuticular surface. Fuxa and Richter (2004) stated that hyphae from *M. anisopliae* conidia entered the host's body with the help of enzymes or mechanical pressure. In the end, the host was covered all over with propagules and the soft parts of the body were penetrated so hyphal growth could be observed outside the host insect's body. External hyphal growth would produce conidia which spread spores into the environment upon reaching maturity, then infect other healthy insects. We concluded that *B. bassiana* and *M. anisopliae* were able to kill *A. gossypii* nymphs. Twenty five isolates of *B. bassiana* and *M. anisopliae* found were almost pathogenic againts them. The most virulent isolate was BPM of *B. bassiana* isolate causing an average of 80.80% mortality. Dead insect hosts infected by *M. anisopliae* showed the same symptoms as those infected by *B. bassiana*, except for the color of the hyphae which was greenish white.

ACKNOWLEDGEMENTS

We would like to thank Riyanto and Cheppy Wati for their assistance in the surveys. Financial support of this research was provided by the Project of Incentive Research Fund, Ministry of Research and Technology, Republic of Indonesia, Budget Year 2010 with Contract Number: 106/ RD-DF/D.PSIPTN/Insentif/PPK/1/2010, 15 January 2010.

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