Antiviral and Antifungal Activity of Biologically Active Recombinant Bouganin Protein from *Bougainvillea spectabilis* Willd

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ABSTRACT

Bouganin antiviral protein (BAP) gene, one of the ribosome inactivating proteins, isolated from *Bougainvillea spectabilis* Willd. was cloned, expressed and the antiviral and antifungal activities were investigated. The full-length bouganin antiviral protein gene was amplified by reverse transcription-PCR using mRNA as template extracted from mature leaves. The coding region of bouganin gene was cloned into prokaryotic expression vector pETDuet-1 after amplification with end to end gene specific primers. The recombinant plasmid was transformed into *Escherichia coli* cells BL21(DE3)pLysS and the expression of BAP gene was induced by isopropyl β-D thiogalactopyranoside (IPTG). Bouganin antiviral protein having a molecular mass of 28 kDa has been isolated from transformed bacterial colonies. Antiviral activity of bouganin was assayed against *Zucchini yellow mosaic virus* (ZYMV) by a mechanical inoculation test. The antifungal activity of purified recombinant protein was tested against pathogenic and non-pathogenic *Rhizoctonia solani*, *Trichoderma harzianum*, and *Fusarium oxysporum* fungi using disc diffusion method. The increased amount of antiviral protein reduced the disease severity caused by ZYMV. The bouganin antiviral protein was inhibited the growth of *R. solani* by 30.7% and of *T. harzianum* by 20% after 72 h compared to control. No growth inhibition was observed for *F. oxyporum*. All plants including controls treated with *in vitro* expressed BAP protein exhibited severe growth reduction compared with negative control (not treated) plants.

Keywords: *Bougainvillea spectabilis*; Antimicrobial protein; BAP gene; Expression; Antiviral and antifungal activities

Bougainvillea spectabilis Willd. Bitkisinin Biyolojik Olarak Aktif Rekombinant Bouganin Proteininin Antiviral ve Antifungal Aktivitesi

ÖZET

Bougainvillea spectabilis Willd. bitkisinden ribozom inaktive eden proteinlerden olan Bouganin antiviral protein (BAP) geni izole edilerek klonlanmış, ifade edilmiş, antiviral ve antifungal özellikleri araştırılmıştır. Bouganin proteinini

kodlayan genin tamamı bitkinin olgun yapraklarından ekstrakte edilen mRNA'dan Reverse Transkripsiyon-PCR yöntemi ile çoğaltılmıştır. Gen, uçtan uca bölgeyi kapsayacak şekilde tasarlanan spesifik primerler yardımı ile prokaryotik ekspresyon vektörü pETDuet-1'de klonlanmıştır. BAP geninin ifadesi rekombinant plazmitin *Escherichia coli* (BL21(DE3)pLysS) hücrelerine transferi sonrası isopropyl β-D thiogalactopyranoside (IPTG) ile teşvik edilmiştir. Molekül ağırlığı yaklaşık 28 kDa olan BAP transforme edilmiş bakterilerden izole edilmiştir. BAP 'ın antiviral aktivitesi *Zucchini yellow mosaic virus* (ZYMV) kullanılarak mekanik inokulasyon yöntemi ile araştırılmıştır. Antifungal aktivitenin belirlenmesi disk difüzyon metodu yardımıyla patojen ve patojen olmayan *Rhizoctonia solani, Trichoderma harzianum* ve *Fusarium oxysporum* funguslari ile araştırılmıştır. BAP uygulama miktarı arttıkça ZYMV'nin neden olduğu hastalık şiddetinin azaldığı tespit edilmiştir. BAP 72 saatlik kontrol uygulaması ile karşılaştırıldığında, *Rhizoctonia solani*'nin gelişimini % 30.7, *Trichoderma harzianum*'un gelişimini ise % 20 oranında inhibe etmiştir. *Fusarium oxysporum*'un gelişiminde herhangi bir inhibisyon etkisi gözlenmemiştir. *In vitro*'da ifade edilen BAP proteininin uygulandığı tüm bitkiler (sadece BAP protieni uygulanan kontrol grubu da dahil) uygulama yapılmayan bitkilere oranla şiddetli gelişme geriliği sergilemiştir.

Anahtar Kelimeler: *Bougainvillea spectabilis*; Antimikrobiyal protein; BAP geni; Ekspresyon; Antiviral ve antifungal aktivite

1. Introduction

In nature, many plants contain proteins with antiviral activity (Barbieri et al 1993). Antiviral proteins (AVPs) are unable to bind the elongation factors. This leads to the interruption of protein translation step and then, it directly inhibits the protein synthesis on ribosomes (Bolognesi et al 2000; Qi et al 2004). Ribosome-inactivating proteins (RIPs) possess site-specific RNA N-glycosidase activity catalyzing the removal of a single adenine base from a conserved loop of the 28S rRNA of eukaryotic ribosomes (Endo et al 1987) and the 23S rRNA of prokaryotic ribosomes (Hartley et al 1991). Some of these AVPs are belong to the family of RIPs (Barbieri et al 1993).

RIPs have been reported in fungi, bacteria, plants, and algae (Girbés et al 2004). RIPs are common among the plants and generally found in families, such as *Poaceae, Euphorbiaceae, Cucurbitaceae*, and particularly to *Caryophyllales* superorder (Grasso & Shepherd 1978; Stirpe et al 1983; Stirpe & Barbieri 1986; Kwon et al 2000). In plants, the presence of the RIPs has been reported to occur in leaves, roots, seeds, and tubers in different concentrations (Stirpe et al 1992).

Based on their functions and structures, the RIPs are commonly classified into two subgroups. Type I proteins, which their molecular masses ranges between 28 and 35 kDa, consist a single polypeptide chain (Stirpe et al 1983). Type II proteins, which is linked to a cell-binding B chain, consist of a catalytically active A chain (Barbieri & Stirpe 1982; Stirpe et al 1992).

AVPs have been the focus of many researches because of their selective toxicity function. Therefore, enzymatic activities of RIPs have been used; in plant defence (Logemann et al 1992; Lodge et al 1993; Madin et al 2000), AIDS (Scadden et al 1998; Donayre Torres et al 2009), therapeutics against tumors (Schnell et al 1996; Wang et al 1998a), anti-fungal activities (Wang et al 1998b) and even biological weapon (Weiner 1996; Christopher et al 1997). RIPs have also been reported to antiviral (Sadasivam et al 1991) and antibacterial (Hakuba et al 1991; Hartley et al 1991) properties.

Bougainvillea spectabilis Willd. is an ornamental plant belonging to Nyctaginaceae family and commonly known Bougainvillea or Great Bougainvillea (Kobayashi et al 2007). A ribosome inactivating protein gene, bouganin, has been isolated from *B. spectabilis*. Based on SDS/PAGE analysis, the molecular mass of single-chain bouganin is estimated approx. 29 kDa (Bolognesi et al 1997). With the present study, we targeted to clone and express the RIP gene from Bougainvillea and test antiviral and antifungal activities by constructing the prokaryotic expression plasmid

encoding BAP gene. The inhibitory effects of the recombinant BAP was documented.

2. Material and Methods

2.1. Plant material

Bougainvillea (*B. spectabilis*) plant obtained from western part of Turkey was grown in a growth chamber at Van Yuzuncu Yil University Department of Plant Protection. Seeds of squash (*Cucurbita pepo* L.) plant were obtained from the regional certified resources and the seedlings were grown in pots containing sterile torf. All plants were grown at 26 °C with a 16 h/8 h light/dark period.

2.2. Test microorganisms

A highly virulent strain of ZYMV was used to test antiviral activities. The virus isolate was maintained on squash (*Cucurbita pepo* L.) seedlings in climate chamber. Viral inoculum was prepared in sodium phosphate buffer (0.01 M, pH 7.2) from systematically infected leaves of *C. pepo.* Three fungal isolates, *Fusarium oxysporum* (pathogen), *Rhizoctonia solani* (pathogen) and *Trichoderma harzianum* (non-pathogen) were used to test for the antifungal assays. The fungal isolates were cultured and maintained on potato dextrose agar (DIFCO, USA) and kept at the temperature of 25 °C. All viral and fungal isolates used in this study were supplied from the Department of Plant Protection of Van Yuzuncu Yil University.

2.3. Isolation and molecular cloning of a full-length BAP gene

The extraction of mRNA was made from the mature leaves of *B. spectabilis* with silica-based method (Foissac et al 2001). Reverse transcription was carried out using a commercial kit (RevertAid First Strand cDNA kit, Vilnius, Thermo-Fermantas-Lithuania). The complete bouganin gene was isolated by PCR amplification. The gene specific primers (B-*Bam* HI-F-5'-*CAGT*GGATCCGAT GGGTTGGTGGGCTATCAT-3' and B-*Sac*I-R-5'-*CAGT*GAGCTCTTAGGCAATGTTTGGCT CTAGT-3') were designed for the amplification

of full length bouganin gene based on registered sequences in GenBank (GenBank access number AF445416) using Vector NTI Software. The primers contained *BamHI* and *SacI* restriction sites and four unrelated nucleotide residues at their 5'end in order to facilitate the cloning into the pETDuet-1vector (Novagen, Darmstadt, Germany). The gene was then sub-cloned into pGEM-T Easy vector (Promega, USA). The following PCR cycle was used to amplify the BAP gene: denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 1 min, annealing at 50 °C for 1 min, and an extension of 72 °C for 1 min, with a final extension of 72 °C for 10 min. A 50 µL of PCR mixture contained; 1 µL of each primer (100 pmol), 1 µL of dNTPs (10 mM each), 3 µL of $MgCl_2$ (25 mM), 2 µL of cDNA, 0.4 µL of Go Taq G2 Hot Start DNA polymerase, 5 µL of 10× reaction buffer and 36.6 µL of DNase free water. The amplified DNA was electrophoresed in 1% agarose gel. DNA bands were purified using a gel extraction kit (Isolate II PCR and Gel Kit, Bioline, Germany).

2.4. Construction of vector for inducible expression of BAP gene and the purification of recombinant His-tagged bouganin protein

BamHI and *SacI* sites of pETDuet-1, containing 6× His Tag coding sequence, were used to construct the recombinant plasmid. The BAP bearing plasmids were then transformed into competent cells of *E. coli* BL21(DE3)pLysS cells using a micropulser (BioRad, USA). Luria-Bertani (LB) broth agar containing ampicillin was used to grow transformed cells at 37 °C overnight. BAP gene bearing clones were determined by colony-PCR, sequence analysis and by restriction endonucleases. One of the sequenced clones was used for further study. Positively identified clone was grown on an LB broth to OD 600 0.6. With a constant shaking at 25 °C for 16 h, the bacterial growth was stimulated by adding IPTG to a final concentration of 0.4 mM. The growth media was centrifuged and the bacterial cells were pelleted and resuspended with proteinase inhibitor (Roche, Germany). After adding 0.5 mL of TrisHCl (pH 7.5), 1 mL of NP40 (10%), 25 µL of $MgCl_2$ (1 M), 7 µL of 2-mercaptoethanol and

20 µL of DNase I (10 U mL^{-1}), the suspension was sonicated four times (3-5 s) in ice, then, the mixture was incubated at 4 °C for 45 min on a shaker. After adding 0.3 g of NaCl (final concentration 0.5 M) the protein extract was ultracentrifuged at 30,000 rpm for 30 min (4 °C). Recombinant His-tagged BAP protein was purified by chromatography on a Ni2+-NTA agarose resin column. After washing the column with TL buffer (2.5 mL 1M Tris-HCl pH 7.5, 1.46 g NaCl, 5 mL 10% NP40, 35 µL 2-mercaptoethanol, 1 mL 1M imidazole pH 8.0) BAP protein was eluted with TE buffer (50% of imidazole and 50% of TL buffer). Fraction containing BAP protein was analyzed by 12% SDS-PAGE electrophoresis gel along with molecular weight markers (Laemmli 1970; Sankian et al 2007). The protein concentrations were determined according to the method of Bradford (Bradford 1976)

2.5. Bioassay of BAP protein for antiviral activity

The purified BAP protein was suspended in 10% DMSO at final concentrations of 2, 4 and 8 µL and used as stock solutions and applied to squash cotyledons by using a sterile pipette tip periodically through 4 days. Then, for each treatment, ZYMV extract (100 µL) was inoculated on squash cotyledons (Schmitt et al 2001). The development of symptoms was observed for three weeks and the symptoms were recorded as scale value. The controls were consisted of "only BAP protein", "only DMSO 10%", "only elution buffer (TE) (50% imidazole pH 8.0+50% TL buffer)", "only ZYMV", and 10% DMSO+Elution buffer. No treated plants were also used as controls (Table 1). For each treatment, the cotyledons of three *C. pepo* plants were used. All of them were tested in three replicates in each run of the experiments.

Antiviral activity of BAP protein was measured by a disease severity index (DSI) assay using *C. pepo* cotyledons and *Zucchini yellow mosaic virus* (ZYMV). A 0 to 5 rating scale was adapted to score the disease severity index (DSI) described by Liu et al (1995), where 0= no symptoms, 1= leaf spotting at least fifty percent of the leaf surface area, 2= leaf spotting at least more than fifty percent of leaf surface area, 3= mottle and mosaics, 4=

Table 1- The groups created for determination of antiviral activity of bouganin protein and control groups

Groups	Treatments
1	10% DMSO+2 µL BAP
2	10% DMSO+4 µL BAP
3	10% DMSO+8 µL BAP
4	10% DMSO+2 µL BAP+ZYMV
5	10% DMSO+4 µL BAP+ZYMV
6	10% DMSO+8 µL BAP+ZYMV
7	Only ZYMV (PC)
8	No treatment (NC)
9	Only 10% DMSO
10	Only TE
11	10% DMSO+TE

severe mottle, mosaics and leaf deformations, 5= severe disease symptoms on leaf surface including shoestring symptom. DSI was calculated based on the Equation 1.

Disease severity index (DSI)= $\Sigma(A \times B)/(T \times D) \times 100$ (1)

Where; A, scale number; B, number of symptomatic leaves; T, total number of leaves; D, highest scale.

Statistical significance of the treated groups mean with that of control groups were analyzed by SAS 9.4 (SAS 2014) package program. The General Linear Model analysis was used to determine the differences between the groups in the study followed by Duncan's multiple range tests to separate means. Differences were considered statistically significant if $P<0.05$. To determine the presence of ZYMV, inoculated squash groups were tested by RT-PCR as described by Özer et al (2012). Before inoculation, the plants were exposed to recombinant BAP protein for 4 days and were kept in a growth chamber for 3 weeks. After BAP application, the squash plants were inoculated with ZYMV in the cotyledon leaf stage. The plants were then scored for ZYMV-induced symptom severity. The presence of viral RNA in the inoculated plants was tested by RT-PCR and fresh and dry weights of plants were measured.

2.6. Antifungal activity of BAP protein

The antifungal activity of BAP was tested *in vitro* by a radial growth inhibition as described by Schlumbaum et al (1986). The test was conducted using sterile 9 cm diameter petri dishes. Mycelial plugs (5 mm in diameter) were placed on to 2 cm aside from the outermost of each plate. A total of 50 μL filter sterilized antiviral protein and TE buffer was loaded onto the filter paper disks. As a group of control, different petri dishes were prepared for each fungus. The plates were incubated at 25 °C for 7 days for which the hyphae grew to outwards from the center. The inhibition effect was observed daily following the treatment (24, 48 and 72 h). The fungal growth was measured (mm) daily and percentage inhibition in growth calculated. Three replicates were formed for each fungus.

3. Results

3.1. Cloning and expression of the bouganin gene

The RT-PCR result revealed that only one specific DNA band, 893 bp in length, was visualized in agarose gel electrophoresis. After cloning in expression vector, the gene was sub-cloned in pGEM-T Easy vector. The purified recombinant plasmids were then sequenced bidirectionally to verify the authenticity of the amplicon. The sequencing report showed that the BAP gene shared 85% identity with other isoforms of BAP sequences from *Bougainvillae* species. The BAP gene has an initiation amino acid methionin (ATG), and terminated by alanin amino acid (GCC). The BAP gene contained a complete open reading frame and had no introns. The sequence obtained in this study has been assigned the GenBank with the accession no. KP096226.

3.2. SDS-PAGE analasys of expressed and purified recombianant His-tagged bouganin protein

After the recombinant plasmid containing an N-Terminal 6× Histidin Tag sequence was transformed into the bacterial cells, the fused His-BAP protein was purified by utilizing Ni-NTA affinity column. Based on SDS-PAGE analysis it was shown that the BAP gene was expressed after

IPTG induction. As expected, a 28 kDa fused BAP protein was observed. A very slight unrelated protein bands were seen on SDS-PAGE gel (Figure 1). The concentration of the purified His-tagged bouganin protein was determined as 46.8 μg mL^{-1}.
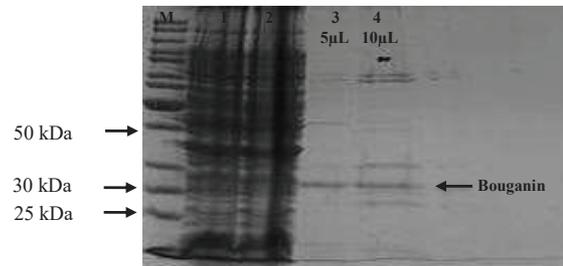

Figure 1- 12% SDS-PAGE electrophoresis gel analysis of the bouganin protein; 1, protein profile of IPTG-inducted *E. coli* containing recombinant plasmids; 2, protein profile of IPTG uninducted *E. coli* bacteria containing the recombinant plasmid; 3 and 4, purified bouganin protein profile; M, molecular protein size marker

3.3. Antiviral activity of BAP protein

The antiviral activity of recombinant BAP protein was examined by symptom expression on *C. pepo*. The results showed that the disease severity index (DSI) was 68.2%, 61.4% and 58.7% with the application of 2, 4 and 8 μL of purified BAP, respectively. The DSI of positive control (PC) was recorded as 72.9%. The most active amount was determined as 8 microliter of BAP protein, which exhibited a DSI of 58.7%. However, by using local lesion analysis Choudhary et al (2008) reported a high level of inhibition (~94%) against *Tobacco mosaic virus* (TMV). Our results demonstrate that the inhibitory activities of BAP against ZYMV are positively correlated with the amount. Therefore, the inhibition of infection and symptom expression by BAP were dose dependent between 2 and 8 μL (Figure 2).

Furthermore, pretreatment of squash leaves with BAP protein for four days before inoculation with ZYMV did not prevent the virus infection. All tested uninoculated squash leaves (controls) were negative while the all inoculated squash leaves
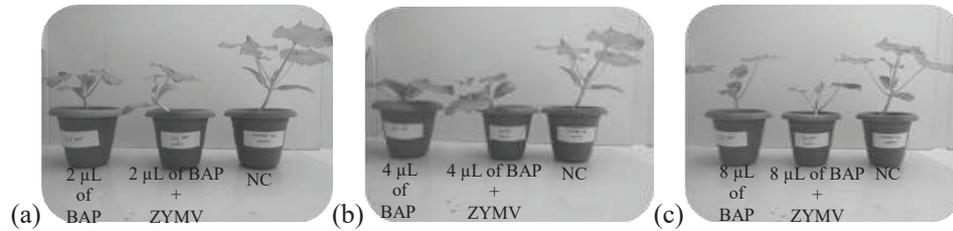
Figure 2- Experiment of bouganin antiviral protein (BAP) activity to *Zucchini yellow mosaic virus* (ZYMV); the cotyledon leaves were pretreated with 2, 4 and 8 µL of BAP in the presence of 10% DMSO for four days then, 100 µL of ZYMV inoculum were inoculated to the same leaves; the only purified BAP of 2, 4, and 8 µL were also kept as controls in the presence of 10% DMSO; NC, negative control

were reacted positive in RT-PCR tests 3 weeks after inoculation. DSI analysis of inoculated squash plants demonstrated that plants treated with 4 µL and 8 µL of BAP showed a significant reduction (P<0.05) in symptom expression compared with controls.

When BAP was applied with 10% DMSO to the leaves of squash plants, a severe reduction (50% or greater) occurred in plant size. The controls involving "only 10% DMSO", "only TE", and "only 10% DMSO+TE" showed no significant changes in plant size as compared to the control group (NC). Fresh and dry weights of BAP treated squash plants were also showed a severe decrease as compared to control plants. The obtained data was evaluated with the standard deviations and found significant statistically (Table 2). These results suggest that BAP protein blocks essential cellular functions needed for growth. The reduction was greater when ZYMV was inoculated after BAP treatment period. In those plants, besides delayed symptom expression as compared with positive controls, a severe growth reduction was also recorded.
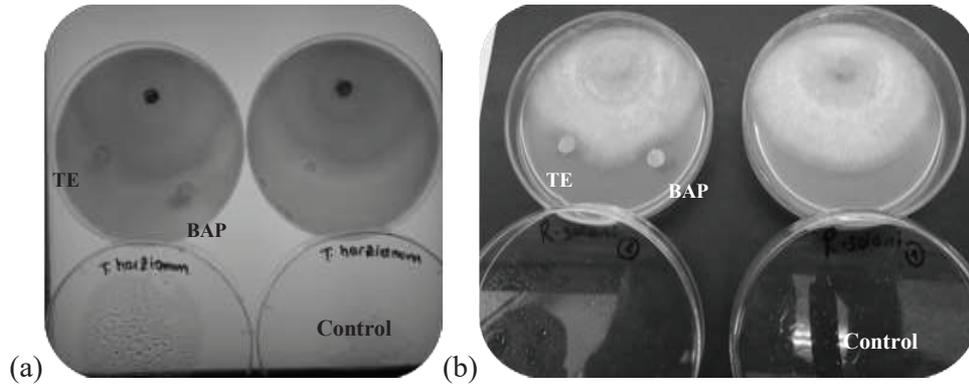
3.4. Antifungal activity of BAP protein

The results of antifungal activity of BAP against various fungi [*F. oxysporum* (pathogen), *R. solani* (pathogen), and a non-pathogenic fungus *T. harzianum,* (data not shown)] are shown in Figure 3. For the determination of antifungal activity, a 50 µL (46.8 µg mL^{-1} in 1 mM TE buffer, pH 7.5) of filter sterilized BAP suspension was used. It was shown that BAP inhibited the two of these fungi (Table 3).

Table 2- Average fresh and dry weights and statistical analysis of the squash plants used in this study

Groups[1]	Dry weight (g) $\left(\overline{X} \pm S_{\overline{x}}\right)$[2]	Fresh weight (g) $\left(\overline{X} \pm S_{\overline{x}}\right)$
NC	4.54±0.12[a]	21.40±0.34[a]
Only ZYMV (PC)	3.87±0.08[b]	14.40±0.23[b]
Only TE	4.60±0.03[a]	21.30±0.21[a]
Only DMSO 10%	4.60±0.20[a]	21.20±0.50[a]
TE+DMSO	4.70±0.10[a]	21.70±0.14[a]
2 µL BAP	3.90±0.11[b]	15.33±0.35[b]
4 µL BAP	3.61±0.24[bc]	14.56±0.34[b]
8 µL BAP	3.40±0.09[c]	13.81±0.79[b]
2 µL BAP+ZYMV	3.72±0.05[bc]	11.17±0.85[c]
4 µL BAP+ZYMV	3.64±0.12[bc]	10.47±1.43[c]
8 µL BAP+ZYMV	3.55±0.12[bc]	7.83±1.50[d]

[1]; a, b, c, d means the difference between the averages with different letters in the same column is significant; [2], mean± standard error of mean; BAP, Bouganin antiviral protein; DMSO, dimethylsulphoxide; ZYMV, Zucchini yellow mosaic virus; TE, Tris EDTAbuffer; PC, positive control; NC, no treatment

Based on time course experiment a moderate mycelia growth inhibition was observed with 50 µL purified BAP for up to 72 h of incubation. Two fungi *R. solani* and *T. harzianum*, exhibited a crescent-shaped zone of inhibition at their mycelial front (Figure 3). BAP was found to be most active on these two. No antifungal activity was detected against mycelial growth of *F. oxyporum*. TE buffer which was used as negative control did not showed any impact on the growth of fungi. Another remarkable finding in this study that bouganin protein was promoted the sporulation of T. *harzianum* (Figure 4).

(a)   (b)

Figure 3- Antifungal activity of BAP against *T. harzianum* (a) and *R. solani* experiment (b); the dosage of the antiviral protein was 46.8 μg mL^{-1}; a crescent-shaped zone of inhibition at the mycelial front is seen; the controls consisted of 1 mM TE buffer, pH 7.5 and untreated separate inoculations of same fungi

Table 3- Inhibitory effect of Bouganin antiviral protein against some fungi in PDA

Fungal agent		The average growth of the fungus mycelium (cm)			Inhibition (%)
		24 h	*48 h*	*72 h*	
F. oxysporum	Control	-	1.9	2.9	-
	Treatment		1.6	2.9	
R. solani	Control	3.7	5.9	6.5	30.7
	Treatment	3.3	4.3	4.5	
T. harzianum	Control	2.8	3.7	5	20
	Treatment	2.3	3.7	4	
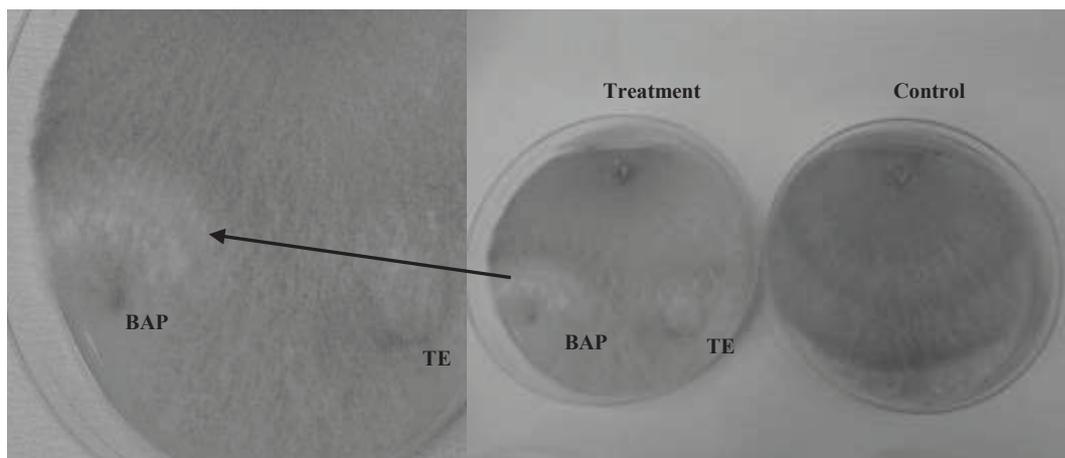
Figure 4- Sporulation of *T. harzianum* at the point where the fungus encounters with BAP protein

4. Discussion

The BAP I gene contains 297 amino acids, phylogenetic analysis of BAP I nucleotide sequence with those of other BAPs, on a consensus length of 893 nucleotide residues, established a maximum homology of 85%.

The *in vitro* tests showed that BAP proteins had activities against ZYMV symptom reduction, but its inhibitory activity on ZYMV infection was not confirmed. The relationship between the concentration of BAP protein and its antiviral activity toward tested virus was not established in this investigation. However, here we report that the inhibitory activity of BAP protein against ZYMV is a dose dependent manner. There was a steady decrease in the DSI with the increase in the amount of BAP for all the replicates tested for ZYMV. The present findings suggest that the antiviral activity of BAP could be related to its amount in diffused cells. It has been reported that the all types of RIPs have antiviral activity against plant viruses (Stevens et al 1981; Hartley et al 1996; Jackman et al 1999). Most of the studies shows that inhibit the symptoms of the virus but the presence or the absence of the virus have not been studied. Although disease severity has been decreased and is also important statically (P<0.05), when BAP protein applied to cotyledons of squash plants did not prevent the virus entry and it did not stop the ZYMV infection. BAP protein exhibits strong antiviral activity particularly toward *Tobacco mosaic virus* (Choudhary et al 2008). BBAP1 protein which derived from another Bougainvillea species (*B. xbuttiana*) is similar with the effect of inhibition showed against *Sunnhemp rosette virus* (SRV) (Choudhary et al 2007).

Antifungal activities of BAP protein have been performed using radial growth inhibition assay in *R. solani, T. harzianum and F. oxyporum.* This protein displayed an inhibitory activity on *R. solani,* and *T. harzianum*, but there was no obvious inhibitory effect against *F. oxyporum*. In our study, the bouganin protein has inhibited the development of *R. solani* (30.7%) and *T. harzianum* (20%) mycelium. BAP protein has not shown any positive or negative effect in *F. oxyporum.* These findings have shown similarity with the results obtained from the studies carried out of Lodha et al (2010) by BBAP1 *(Bougainvillea xbuttiana* antiviral protein). In parallel, the BAP protein derived from *B. spectabilis* plant has inhibited mycelial growth of the *Sclerotium rolfsii* which is a plant pathogen and determined to reduce the growth in 3 days (Abbas 2007). In other study, conducted by Barbieri et al (2006), the RIP gene isolated from *Cucurbita moschata* plant inhibited the growth of two strain of *Phytophthora infestans.* Roberts & Selitrennikoff (1986) reported that the barley RIP inhibited *Trichoderma reesei* growth on agar plates. Besides, the abundant maize kernel ribosome-inactivating protein (RIP1) has been shown to have antifungal activity against *Aspergillus nidulans* (Nielsen et al 2001). In our trials, the BAP antiviral protein has encouraged sporulation of *T. harzianum.*

Interestingly, we have found a unique characteristic of BAP protein that distinguishes it from known types of RIPs. We found that the BAP treatment along with 10% DMSO causes a severe size reduction in squash. It has been hypothesized that once the BAP reaches the plant cells, it may depurinate the host plant ribosomes and arrests the protein synthesis necessary for the growth. In eukaryotes, it has been well documented that the ribosome inactivating trait of RIPs is responsible for inhibition of protein synthesis (Gessner & Irvin 1980; Irvin 1995). To date, the potential size reduction effect of RIPs in plants has not been investigated. To our knowledge, this is the first time that plant size reduction activity has been reported for BAP protein which has been grouped among type I RIPs. The RIPs are known to have the ability to inactivate fungal, bacterial, mammalian and plant ribosomes (Girbés et al 2004). RIPs act on ribosomes to inhibit polypeptide chain elongation (Olsnes & Pihl 1980; Barbieri & Stirpe 1982; Irvin 1983) and thereby arresting protein synthesis and causing cell death (Endo & Tsurugi 1988). For better understanding of the molecular mechanisms of BAP blockage of cellular functions that are needed for growth, would be the focus in the future research.

With the present study, we cloned and expressed the RIP gene from Bougainvillea and tested antiviral and antifungal activities by constructing the prokaryotic expression plasmid encoding BAP gene. Here, we describe the isolation and properties of BAP antiviral protein with a molecular mass of 28 kDa from *B. spectabilis*. Full-length DNA encoding bouganin antiviral protein gene was generated using gene specific forward and reverse primers and cloned into pETDuet-1 expression vector. In conclusion, purified *E. coli*-expressed BAP antiviral protein from *B. spectabilis* has multifunctional activity against ZYMV, *R. solani*, and *T. harzianum*. This is the first report of the plant growth reduction effect of BAP.

Acknowledgements

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