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Full Length Article

Endophytic *Trichoderma* Species of Palu Valley Shallot Origin with Potential for Controlling Purple Blotch Pathogen *Alternaria porri*

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Abstract

Endophytic strains of *Trichoderma* can be used as an alternative to chemicals to control plant disease. Here, the work report on isolation and identification of *Trichoderma* from shallot (*Allium cepa* var *agregatum*) in Palu Valley, the endophytic capability of these fungi to colonize plant tissues, then *in vitro* and *in vivo* applications of *Trichoderma* to control the pathogen of shallot purple blotch disease, *Alternaria porri*. Of three isolated, two strains (T1FLS and T3RZR) were characterized as *T. asperellum* and one isolate (T2RZS) as *T. harzianum*. All were recovered from root, stem and leaf tissues when the fungi were reintroduced into young shallot by root inoculation. *In vitro* application indicated that these three strains inhibited *A. porri* colony by 74.6, 71.4 and 70.9%, respectively, nine days of post-dual culture on PDA medium. *In vivo* trial showed that pre-treatment of shallot with *Trichoderma* through roots could inhibit purple blotch intensity by 39.3, 49.1 and 3.6%, respectively, 35 days after *A. porri* inoculation. Therefore, the study results demonstrate the presence of endophytic *Trichoderma* in Palu Valley shallot crops, and especially *T. asperellum* strain T3RZR could potentially be used as bio-fungicide to control the purple blotch disease in the field.

Keywords: Endophyte; Inhibition; Purple blotch; Shallot; *Trichoderma*

Introduction

Shallot of Palu Valley origin, Central Sulawesi, Indonesia known as Palu local variety *Allium cepa* var. *agregatum* has high commercial value due to its use as raw material for the fried shallot. Several fungal pathogens attack this crop and cause severe losses in many production areas including in the region (Ilhe *et al.* 2013; Fadhilah *et al.* 2014; Hekmawati *et al.* 2018). The major diseases of shallot include basal bulb rot (*Fusarium oxysporum*), purple blotch (*Alternari porri*), anthracnose (*Colletotrichum gloeosporiodes*) and downy mildew (*Peronospora destructor*) (Adiyoga *et al.* 2014; Hidayat and Sulastrini 2016; Sari *et al.* 2016; Akhtar and Javaid 2018). Small farmers typically produce shallot with a high frequency of pesticide applications (Shahabuddin *et al.* 2012; Joko *et al.* 2017). The field observation indicates that a high pesticide usage on shallot has resulted in several ecological and environmental consequences such as pest and disease resistant, degradation potential of soil quality, and decrease of natural enemies (Basuki 2011; Nelly *et al.* 2015; Joko *et al.* 2017). For reducing these negative impacts, biocontrol appears to be one of the few viable approaches. Many researchers have investigated using *Trichoderma* as a durable, plant-based, biocontrol alternative for the management of shallot diseases, in the short and or long-term period.

Trichoderma species (teleomorph *Hypocrea*) are considered mostly to be common soil inhabitants, saprophytes and parasites of other fungi (Chet *et al.* 1998). As, a parasite, these fungi can inhibit plant pathogens, especially in the soil or on plant roots, through their high antagonistic and mycoparasitic potential (Viterbo and Horwitz 2010). *Trichoderma* is also capable of more intimate associations with plant root systems in what has been characterized as an opportunistic, avirulent symbiotic relationship. The critical phase of this association is the penetration of the fungus into the outer layers of cells of plant roots, where it persist. This association induce metabolic changes in plant that increase resistance to a wide range of plant-pathogenic microorganisms and viruses, known as systemic resistance occurring through jasmonic acid/ethylene signalling pathway in a way similar to the rhizobacteria-ISR (Harman *et al.* 2004; Shoresh *et al.* 2005; Loon 2007). Some strains have also shown to be among the most abundant avirulent endosymbionts in the living sapwood and leaves of trees such as *Cola* spp., *Herrania* spp., *Theobromae* spp., and *Heveae* spp. (Evans *et al.* 2003;

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Holmes *et al*. 2004; Chaverri *et al*. 2011). With the capability to deploy systemically in cacao tissue when applied into the soil, *Trichoderma* are capable of controlling the fungal disease effectively infesting above the ground such as vascular streak dieback (Rosmana *et al*. 2018a, b). The use of *Trichoderma* as a biocontrol agent against shallot diseases, especially purple blotch is still limited. It is apparently due to the fact that farmers are more confident with the application of synthetic fungicides in their crops. This purple blotch, which is caused by *A. porri* is one of the most destructive diseases infesting the genus *Allium* and widespread in the world. *Alternaria* spores germinate on leaves and produce a small water-soaked spot that turns brown. Then, this spot enlarges into the zonate elliptical lesion with purplish colour. The lesion may merge or become so numerous that can kill the leaf. Lesions may also form on seed stalks and floral parts of the seed, and affect seed development (Schwartz 2011). Production losses due to this disease are estimated at 50% until 70% (Schwartz 2011; Farid 2012). Here, the study focused on isolation and identification of *Trichoderma* from shallot in Palu Valley, the endophytic capability of these fungi to colonize plant tissues, then *in vitro* and *in vivo* applications of *Trichoderma* to control *A. porri.*

Materials and Methods

Sources of *Trichoderma* isolates and *Alternaria porri*

Trichoderma species were obtained from roots and leaves of shallot grown in Olobuju village, Sigi Biromaru District, Sigi Regency, Central Sulawesiis, Indonesia also known as Palu Valley. For isolation, shallot with low, moderate, high application of pesticides was sampled. Low was an application with just herbicide, and natural pesticide, moderate was an application with less than ten times, and high was with more than ten times per season. These roots and leaves were surface sterilized by sequential immersion in 2% sodium hypochlorite, 70% ethanol and sterilized water for two minutes respectively (Arnold *et al.* 2003) and then placed in Petri dishes containing 20 mL potato dextrose agar (PDA). Purification of isolates was done through removing the growing mycelia to a new PDA medium.

Alternaria porri as pathogen was as well obtained from the same place mentioned above. Leaves showing symptoms of purple blotch were cut into 2 cm sections and placed in Petri dishes containing sterile filter paper. The growing fungus was then transferred to PDA medium in Petri dishes, and this source was used for the antagonistic study with *Trichoderma*. While for the purpose of direct infection onto shallot, leaves infected severely by pathogen were crushed in a mortar. With addition of sterile distilled water, the suspension was filtered by using the muslin cloth to separate conidia. Then, these conidia were ready for immediate use in inoculation into healthy shallot.

Identification of *Trichoderma* isolates

Three *Trichoderma* strains were grown in potato dextrose broth in 100 mL volume Erlenmeyer for five days at 27–29°C. Then their mycelia were harvested and dried using sterile, absorbent, paper filters. By using these mycelia, genomic DNA extraction was performed as reported previously (Dodd *et al.* 2002). Polymerase chain reaction for amplification of internal transcribed spacer (ITS) region in first and third isolates and nuclear, large subunit rDNA (LR) in the second isolate was performed with 35 cycles of pre-denaturation for 120 s at 95°C, denaturation for 60 s at 94°C, annealing for 30 s at 50°C, elongation for 90 s at 72°C, and post-elongation for 5 min at 72°C. ITS 4 and ITS 5 and LROR 5 and LR 5 were used as primers to amplification these ITS and LR. An amplicon of 600 bp and 900 bp, respectively, was obtained and sequenced. Sequencing and assembly were done at Axil Scientific, Singapore.

Antagonistic screening

Three *Trichoderma* strains were screened for antagonistic ability using a dual culture method as previously described (Nurbailis *et al.* 2015). A 0.5 cm diameter of inoculums taken from a freshly sporulating of The *Trichoderma* isolate was placed at one edge of a 9 cm diameter PDA plate, and the same size of *A. porri* inoculum was set at other side offering a distance of 3.0 cm. Five replicate plates were prepared and incubated at room temperature (27°C–29°C), therefore the total of 15 plates. The percentage of *A. porri* inhibition was calculated by using the formula of $R = (r_1 – r_2)/r_1$ where R is the percentage of inhibition, r_1 is the radius of the colony that is not dealing with *Trichoderma* and r_2 is the radius of the colony facing *Trichoderma*.

Assessment of endophytic ability

All *Trichoderma* isolated from shallot leaves and roots were tested for their ability to colonize young plant. Forty bulbs were planted in poly-bags containing about 250 g soil and then placed in the greenhouse. After three days, 1×10^6 mL^{-1} spores of each isolate were inoculated into ten shallots through the soil, respectively. For determination of endophytic *Trichoderma* presence in shallot tissues, five plants were sampled one week and three weeks post-inoculation. Roots and stems were cut into 1 cm section and leaves into 1 cm^2 piece. Then, these all were sterilized in 2% sodium hypochlorite, 70% ethanol and vigorously washed several times in sterile distilled water before being placed onto PDA in Petri dishes. In each Petri dish contain five sections or pieces and after incubation at room temperature, the presence of *Trichoderma* in these sections or parts was observed. Its colonization was calculated by using the formula of $C = p/5 \times 100\%$, where C is colonization and p is the number of sections or pieces presenting the occurrence of *Trichoderma*.

Efficacy screening for *Alternaria* purple blotch

The trial was done in the greenhouse with the treatment of three *Trichoderma* isolates. Each treatment consisted of five shallots, so a total of 20 plants, including control. These shallots were applied, respectively through soil drenching with 10 mL of *Trichoderma* suspension containing 10^6 conidia at three days post-planting and through foliar spraying with 10 mL of *A. porri* suspension containing 10^6 conidia at seven days post-planting. The disease was evaluated weekly after inoculation by the pathogen by assigning an intensity score of purple blotch damage to the whole leaf as follow: 0, no infection; 1, < 25 % leaf infected; 2, 25–50% leaf infected; 3, 50–75% leaf infected; 4, >75% leaf infected. For each *Trichoderma* treatment, mean disease intensity was calculated as follow $I = (n_1 \times 0 + n_2 \times 1 + n_3 \times 2 + n_4 \times 3 + n_5 \times 4)/ (N \times Z)$ where I is intensity, n_1- n_5 is number of leaves uninfected or infected in each score, N is total of leaves observed, and Z is the highest score found.

Statistical analysis

Percentage inhibition of *Alternaria porri* by *T. asperellum in vitro*, colonization of *Trichoderma* in shallot tissues and intensity of disease on shallot were analyzed without any data transformation. The least significant difference was then used for evaluating significant differences between the treatment means.

Results

Identification of *Trichoderma* from shallot

A total of three isolates from shallot were obtained from root and leaf tissues. These isolates were identified at the species level by analysis of their internal transcribed spacer (ITS) region and nuclear, large subunit rDNA (LR) (Table 1). Isolate T1FLS originating from a plant treated moderately with pesticides had a 99% homology with *Trichoderma asperellum* strain ZWPBG2. Then, Isolate T2RZS from plant treated with the high frequency of pesticides had a 99% homology with *T. harzianum* strain XZN202-1. While isolate T3RZR from plant applied with the low frequency of pesticides had a 100% homology with *T. asperellum* strain HNZZ1006.

Antagonistic study

Dual culture essay of *Trichoderma* and *A. porri* in PDA medium indicated that three isolates cold inhibit the growth of the pathogen. By isolate T1FLS, the inhibition five, seven, and nine days post-dual culture by strain T1FLS was 48.1, 74.6 and 76.0%, by strain T2RZS was 70.9%; was 38.7, 70.9 and 73.6%, and by s 71.4% and by strain T3RZR was 36.8, 36.8 and 73.6%, respectively (Fig. 1). Statistical analysis indicated that the inhibition capacity of three strains against pathogen was not significant ($P \leq 0.05$), except at five days post-dual culture, where the lowest found by isolate T3RZR.

Endophytic assessment

Three isolates of *Trichoderma* were found from all inoculated shallot tissues at one and three weeks post-inoculation through the soil. In the uninoculated shallot, these *Trichoderma* were either not detected or occurred at a level that was far below that observed in the inoculated shallot (Fig. 2). *Trichoderma asperellum* strain T1FLS was capable of moving from site of inoculation in the soil to roots, stems, and leaves and reached colonization of 40.0, 20.0 and 33.3% one-week post-inoculation and 66.7, 20.0 and 33.3%, respectively three weeks post-inoculation. Then, *T. harzianum* isolate T2RZS reached as well root, stem, and leaf tissues with the colonization of 46.7, 20.0 and 33.3% one-week post-inoculation and 60.0, 20.0 and 33.3%, respectively three weeks post-inoculation. While, *T. asperellum* strain T3RZR with the colonization of 66.7, 20.0 and 33.3% one-week post-inoculation and 73.3, 26.7 and 40.0%, respectively three weeks post-inoculation (Fig. 2). Therefore, the highest colonization of these three *Trichoderma* strains was in roots, and the lowest was in stems.

Screening for *Alternaria* purple blotch

Screening experiments were conducted for 43 days to estimate the potential of *T. asperellum* isolate T1FLS, *T. harzianum* isolate T2RZS, and *T. asperellum* isolate T3RZR. Disease intensity was 1.8, 2.8, 5.4 and 6.2% in shallot uninoculated with three isolates of *Trichoderma* 15 days, 22 days, 29 days, and 36 days post-inoculation by *A. porri*, respectively. Treatment with isolate T1FLS, disease intensity, decreased to became 1.7, 2.6, 2.7 and 3.8%, respectively and statistical analysis indicated that this disease intensity at the last two observations was significantly different ($P \leq 0.05$) with the control. After treatment with strain T2RZS, the disease intensity became 2.0. 3,4, 4.7 and 6.0% and no of these intensities were significant with control. While the disease intensity in shallot treated with isolate T3RZR became 1.3, 1.7. 2.4 and 3.2%, respectively and significantly different with control was observed at 29 days and 36 days post-infection. The efficacy of these three isolates in the last observation was 39.3, 3.6 and 49.1%, respectively (Fig. 3).

Discussion

The results reported here are the first assessments of endophytic *Trichoderma* presence in shallot crops in Central Sulawesi, Indonesia. Three strains including *T. asperellum* strain T1FLS, *T. harzianum* strain T2RZS, and *T. asperellum* strain T3RZR were discovered from plant

Table 1: Sources and identification of *Trichoderma* strains from shallot in Palu Valley, Sigi Regency, Central Sulawesi in this study

No	Source	Strain number	Identified as	Homology with	Percent of homology	Accession number
1	Leaf	T1FLS	*T. asperellum*	*T. asperellum* strain ZWPBG2	99	KR868290.1
2	Root	T2RZS	*T. harzianum*	*T. harzianum* strain XZN202-1	99	MF109019.1
3	Root	T3RZR	*T. asperellum*	*T. asperellum* strain HNZZ1006	100	JQ040317.1

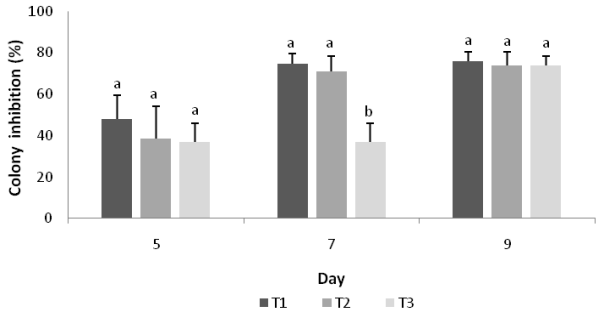

Fig. 1: Radial growth inhibition of *Alternaria porri* in PDA medium five, seven, and nine days of post-dual culture with three *Trichoderma* isolates. T1, *T. asperellum* strain T1FLS; T2, *T. harzianum* strain T2RZS; T3, *T. asperellum* strain T3RZR. Means of inhibition at the same time, followed by the same letter are not significantly different, according to LSD ($P \leq 0.05$)

applied with the high, moderate, and low frequency of pesticides, respectively. This discovery indicated that *Trichoderma* could survive to the application of pesticides. Three suggestions were proposed for their survival capability in the toxic environment. Firstly, the low accessibility of pesticides into shallot tissues. Therefore, it seems that these pesticides have little impact on the presence of endophytic microorganisms. Secondly, *Trichoderma* was tolerant of pesticides. *In vitro* study indicate that the fungicides wettable sulphur and copper oxychloride don not affect the mycelial growth of *Trichoderma,* even in high concentration. The same results was also observed with the insecticides diazinon, cypermethrin, and imidacloprid, (Mohamed and Radwan 2017; Silva *et al.* 2018). Thirdly, contrarily to the *Trichoderma*, other microorganisms probably were reduced by pesticides and for this reason only fungi *Fusarium*, *Gliocladium*. *Penicillium* and *Aspergillus* were identified from shallot (Ratnawati, unpublished data). Fungal species presence in plant tissues is regulated by the chemistry and the interspecific competition among fungi (Fang *et al.* 2013). Therefore, a limitation of fungi community supports probably for the survival of *Trichoderma.*

All *Trichoderma* can be detected from the root, stem, and leaf tissues of shallot after application through the soil. It was proven that the three isolates could move from the site of inoculation into these tissues as endophytes. Their endophytic capacity resembles *T. asperellum* isolate ART-4 on cacao seedling (Rosmana *et al.* 2018b). Endophytic fungi, usually, infect and live within living plant tissues without causing any manifestation of disease symptoms or external structural modification. They can grow within

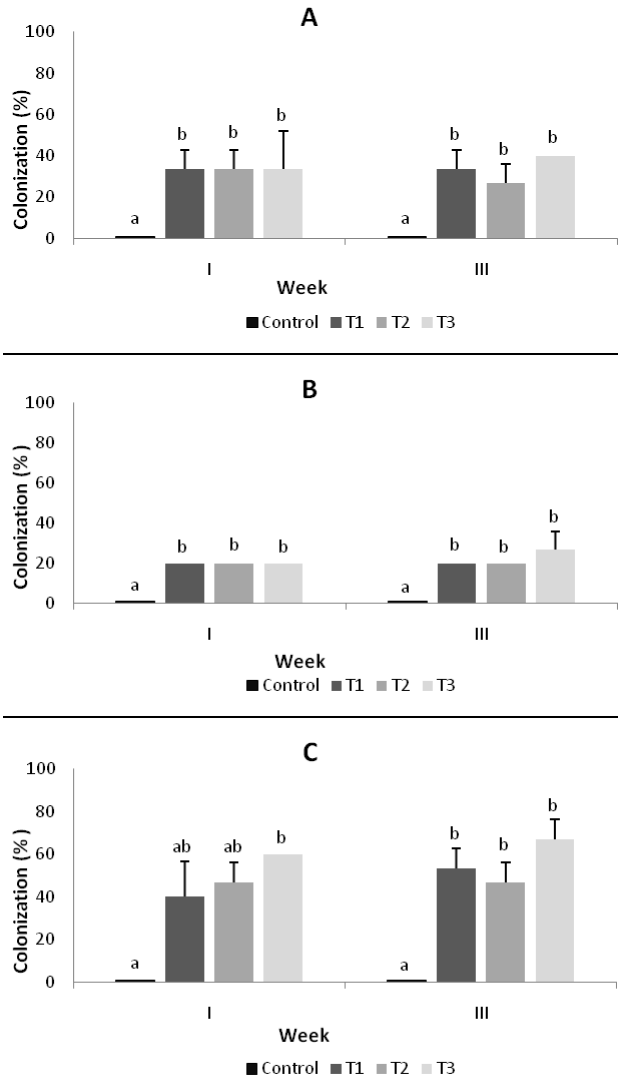

Fig. 2: Colonization of three *Trichoderma* isolates in leaf (**A**), stem (**B**), and root **C**) tissues one week and three weeks post-inoculation through the soil. T1, *T. asperellum* strain T1FLS; T2, *T. harzianum* strain T2RZS; T3, *T. asperellum* strain T3RZR. Means of colonization at the same time, followed by the same letter are not significantly different, according to LSD ($P \leq 0.05$)

roots, stems or leaves, and sometimes emerge to produce spores at plant-tissue senescence (Rodriguez *et al.* 2009; Pancher *et al.* 2012). Endophytes are classified in four classes, each defined by the plant tissues it infects, and its transmission. First-class endophytes (C-endophytes) are members of the Clavicipitaceae. These endophytes infect grasses and are seed-borne, growing from a seed into leaves.

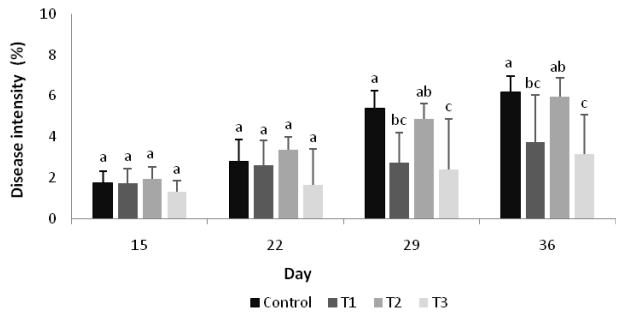

Fig. 3: The intensity of purple blotch disease after treatment by *T. asperellum* isolate T1FLS, *T. harzianum* isolate T2RZS, and *T. asperellum* isolate T3RZR 15 days, 22 days, 29 days, and 36 days post-inoculation of *Alternaria porri*. T1, *T. asperellum* strain T1FLS; T2, *T. harzianum* strain T2RZS; T3, *T. asperellum* strain T3RZR. Means of intensity at the same time, followed by the same letter are not significantly different according to LSD ($P \leq 0.05$)

Second, third, and fourth-class endophytes belong to many taxonomic groups except the Clavicipitaceae (NC-endophytes). These endophytes are not borne by seed and occur in the majority of plant groups except grasses (Arnold and Lutzoni 2007; Rodriguez *et al.* 2009).

The production of antimicrobial compounds and the feeding on a fungus by another organism are mechanisms whereby *Trichoderma* offer protection to plant against pathogens. The two tools are called as antibiosis and mycoparasite, respectively (Chet *et al.* 1998; Harman *et al.* 2004). In this study, the three strains showed almost the same efficacy in inhibition against *A. porri* in nine days. However, the process of inhibition by *T. asperellum* isolate T1FLS and *T. harzianum* isolate T2RZS was relatively fast while by isolate *T. asperellum* isolate T3RZR was relatively slow. This difference indicated the distinction in the mechanism of inhibition. The strain T1FLS and T2RZS have a mechanism of antibiosis, while the isolate T3RZR current mechanism of mycoparasite.

Alternaria spore germinates, penetrates the surface of leaves and produces a small, water-soaked spot that expands rapidly into the oval, brown to purple blotches, several centimeters long. If the symptom grows around the leaves or merge, the parts above the symptom become wilt, collapse, and die (Aveling *et al.* 1994; Schwartz, 2011). Application of *T. asperellum* isolate T1FLS, *T. harzianum* isolate T2RZS, and *T. asperellum* isolate T3RZR through soil inoculation reduced disease intensity by 39.3, 3.6 and 49.1%, respectively. Therefore, *T. asperellum* showed more superior to *T. harzianum*, and *T. asperellum* isolate T3RZR tended more efficacy than isolate T1FLS in inhibition of the purple blotch disease. The superiority of isolate T3RZS has correlated apparently to more capacity in colonizing root and also stem and leaf tissues compared to two other strains. The ability of *Trichoderma* to colonize roots has been used as a selectable trait (Harman *et al.* 2004). However, its presence at the same time in leaves and stems would offer localized effects either via direct pathogen inhibition or via localized induction of host defensive pathways (Aneja *et al.* 2006; Bailey *et al.* 2006). Since *A. porri* infect upper part of shallot, the control hypothesis of three strains of *Trichoderma* against the pathogen is three mechanisms. The first mechanism is due to resistant induction through interaction these fungi with roots (Harman 2011; Hermosa *et al.* 2013). The second is caused by localized induction of resistant. The third is direct action through their antibiosis or mycoparasitic capacity in infection site.

Conclusion

Two strains of *T. asperellum* (T1FLS and T3RZR) and one strain of *T. harzianum* (T2RZS) were characterized from shallot crop in Palu Valley, Sigi Regency, Central Sulawesi. Among these three, the T3RZR strain showed the same capacity in inhibition against *A. porri* with other two isolates *in vitro*. However, *in vivo* application, this *Trichoderma* tended to exhibit more capacity to colonize the root, stem, and leaf tissues and to inhibit purple blotch disease than the two others. Therefore, T3RZR strain could potentially be used as bio-fungicide to control the purple blotch in the field. Some farmers in the region are aware that the use of pesticides in high frequency could be hazardous for their health and environment. For supporting their wish, the application of the strain combined with some method of cultural practices is on the way in Palu Valley.

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