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OVERPRODUCTION OF NEUTRAL PROTEASE IN *BACILLUS SUBTILIS* 168 THROUGH SITE-DIRECTED MUTATION FOR BIOCONTROL OF *MELOIDOGYNE INCOGNITA*

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SUMMARY

The root-knot nematode (RKN), *Meloidogyne incognita*, poses significant challenges for many important crops. Identified distinctive bacterial proteases have emerged as potential nematicides. In this study, the *npr*E from *Bacillus subtilis* 168 encoded the neutral metalloprotease, effectively enhancing it through a site-directed mutation. The enzyme-specific activity of *pro*118, secreted by the modified strain *BS*118, reached 215.4 U mg^{-1}. It was twice that of pro168 produced by the wild-type strain *BS*168, which recorded an enzyme-specific activity of 101.6 U mg^{-1}. The protease, *pro*118, caused the highest mortality in *M. incognita* juveniles, J_2S (91.3% to 84.5%) after 48 h of in vitro treatment. Furthermore, when simultaneously applying *pro*118 with the nematode infection, it notably decreased the number of J_2S, root galls, and egg masses/root systems by 19.5%, 18.8%, and 26.2%, respectively, compared with its application one week after the nematode infection. This consistent trend also manifested in the plant shoot length and fresh weight enrichment, which showed an increase of 9.7% and 14.7% in the first treatment versus the second. In conclusion, the study asserts that applying neutral metalloprotease as a bioagent to biocontrol *M. incognita* is a promising approach for mitigating the impact of this agricultural pest.

Keywords: Site-directed mutation, *Bacillus subtilis* 168, neutral metalloprotease, *Meloidogyne incognita*, nematicidal agents, growth parameters, eggplant

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Key findings: The site-directed mutation, which involves replacing the native promoter with another constitutive one, is a promising approach for constructing strains with higher protease modifications. This method's subsequent employment as a bioagent against *Meloidogyne incognita* infection led to significant plant growth and health improvements.

INTRODUCTION

One of the biggest challenges in crop production, particularly in Egypt, is the loss caused by root-knot nematodes, *Meloidogyne spp*. These parasitic nematodes are highly destructive and have a vital impact on the growth of Egyptian vegetables (Mohamed *et al.,* 2021). Managing nematodes is more complex than other pests due to their mode of attack on plant parts and their habitat in the soil. Managing nematodes had chemical nematicides and bioagents as the current strategies (Singh *et al.,* 2017). However, the extensive use of chemical nematicides has unfortunately led to environmental and health concerns.

Meanwhile, the bioagents application has become prevalent but has not received much attention due to insufficient knowledge about the mechanisms of how bioagents affect nematodes. Likewise, another reason is a lack of dose optimization in the field, particularly in the presence of natural soil microflora that may react positively or negatively to the bioagents. Most of the microorganisms exploited as nematicidal bioagents are *Bacillus spp.* (Li *et al.,* 2023).

A discovery revealed that bacterial bioagents could aid nematode control in two ways: 1) through bacterial culture that contains cells and their secreted metabolites and 2) through their purified secondary metabolites and enzymes. Among these enzymes, proteases have reached extensive studies as a nematicidal factor against RKN (Ramadan and Soliman, 2020). The function of these proteases is to break down proteins that make up the cuticle of the nematode (Ruiz *et al.,* 2014). According to Darwesh *et al.* (2019), most of the studied proteases as nematicidal factors are alkaline serine proteases of microbial origin. Although neutral metalloproteases (NMP) have most investigations, they did not prove effective against nematode species other than *M. incognita* as serine proteases. According to Tian *et al.* (2007), it could be due to their lower efficiency as nematicidal factors. The results of these studies could be due to insufficient enzyme dosage adjustment or the distinct behavior of metalloproteases, which rely on the molecular structure of enzymes and how they interact with the proteins in the nematode cuticle.

Several studies have explored the impact of rhizobacteria on plant health (Mohamed *et al.,* 2021; Khanna *et al.,* 2019). Microorganisms directly affect plants by supplying essential nutrients, such as nitrogen, phosphorus, potassium, and other minerals. Likewise, they indirectly improve plant health by producing antagonistic substances, including secondary metabolites and enzymes, and inducing resistance to pathogens. Rhizobacteria can promote developing root colonizers and act as environmental protectors, enhancing plant health. This study focused on the role of NMP as a single factor to enhance plant parameters. However, this research acknowledges previous investigations on other factors, such as rhizobacteria, which play a significant role in the process. In this research, eggplant was primarily the sample model plant for investigating the efficacy of NMP in controlling the infection caused by the *M. incognita* J_2S and enhancing plant parameters.

The research objective was to utilize a strain of *Bacillus subtilis* subsp. subtilis strain 168 (*BS*168) as a host for screening neutral metalloproteases (NMP) as a potential nematicidal agent. It also sought to assess the impact of these NMPs on plant parameters by examining the biochemical characteristics of the plants. Accomplishing this had the investigators implement site-directed mutations to increase the extraction of extracellular NMP in *Bacillus subtilis* – a safe bacterium devoid of endotoxin. Developing a genetically modified strain proceeded by

inserting expression cassettes to encode heterologous proteins through these features: the complete genome of *B. subtilis* is readily available (Barbe *et al.,* 2009), and it is a well-known process of encoding NMP by the monocistronic *apr*E gene and secreted in large quantities during the stationary phase of culture growth.

The overproduction of the target component through genetic engineering in microorganisms can offer significant advantages. However, releasing these modified strains into nature without thoroughly probing potential side effects presents environmental risks. Hence, this study's main objective was to assess the effectiveness of the overproduced NMP from modified strains as a nematicidal agent against *M. incognita* J_2S and as an inducer for plant health.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids

This investigation utilized bacterial strains and plasmids comprising *E. coli* DH5α and serving as the cloning and plasmid preparation host. The employed strain *Bacillus subtilis* subspecies subtilis 168, accession no. AL009126.3 (*BS*168), for targeted gene isolation, served as a host for genetic modification. Transformant bacterial strains cultivated in a Luria-Bertani (LB) medium (Bertani, 1951) received ampicillin and neomycin supplements (Sigma, St. Louis, MO). Ampicillin and neomycin application had concentrations of 50 µg/ml and 20 µg/ml, respectively, based on the vector used and antibiotic selection criteria. The genetic attributes of the bacterial strains and vectors, whether purchased or constructed, are available in Table 1.

Protease production and activity estimation

The screening and qualitative assay of protease activity in wild and modified strains progressed on a gelatine agar plate. For quantitative analysis, the strains of *BS*168 and *BS*118 cultured on LB medium for 48 h had a temperature of 37 °C under shaking conditions of 200 rpm. After incubation, the supernatant became a source of protease to determine proteolytic activity, following the method of Pant *et al.* (2015).

Table 1. Bacterial strains and plasmids used in this study.

Bacterial strains	Description	Source
Bacillus subtilis subsp. *subtilis* strain 168	$trpC^2$, sfp^0	Lab stock
E. Coli DH5α	F^- *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20* φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) U169, hsdR17($r_K^- m_K^+$), λ^-	Lab stock
Plasmids	**Description**	**Source**
PGEM-T Easy	Cloning vector, Ap^r	Promega
pBG106	*εpbp*, P_{repU}-*neo*, *εfenF*	(Leclère *et al*., 2005)
pMG112	1340 bp *SalI* and *NotI* $p_{repU-neo}$ fragment from pBG106 cloned into pGEM-T Easy	Lab stock
pMG115	576 bp *ylaA* gene fragment of *B. subtilis* 168 cloned into pGEM-T Easy	This study
pMG116	658 bp *nprE* gene fragment of *B. subtilis* 168 cloned into pGEM-T Easy	This study
pMG117	*YlaA* gene fragment *SalI* and *SacI* double digested and inserted into pMG112	This study
pMG118	*NprE* partial gene fragment *NcoI* and *SphI* double digested and inserted into pMG117	This study

Protease overproduction using site-directed mutation protocol

Primer design

Substituting the promoter of the NMP-encoding gene, P*nprE*, resulted in the construction of a dedicated cassette comprising fragments of the *yla*A and *npr*E genes. The nucleotide sequences for both genes received locus tags "BSU_14710" and "BSU_14700" in the GenBank, respectively. Developing the primers depended on these nucleotide sequences, considering the upstream and downstream regions of P*nprE*. Two design sets of primers had the first set consisting of a sense primer (*ylaA* fwd) and an anti-sense primer (*yla*A rev), featuring artificial restriction sites *Sal*I and *Sac*I, aimed to amplify a partial *yla*A fragment. The second set, comprising *nprE* fwd and *nprE* rev primers with artificial restriction sites *Nco*I and *Sph*I, was designed for PCR amplification of a partial *nprE* fragment. These primers appear in Table 2.

DNA manipulation and PCR amplification

The extraction of genomic DNA used the Wizard® Genomic DNA Purification Kit from Promega (Madison, WI, U.S.A.). The PCR amplification procedure included an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, and hybridization at 50 °C for 30 s for both genes (*ylaA* and *nprE* fragments). Subsequently, an elongation step ran at 72 °C for 45 s, with the final elongation steps concluding at 72 °C for 10 min.

Preparation of competent cells

The production of competent cells derived from *E. coli* DH5 followed the procedure outlined by Yang *et al.* (2022), and competent cells for *BS168* had prompt preparation following the protocol described by Shi *et al.* (2013).

Construction of the modular cassette

This procedure aimed to substitute the native promoter *Pnpr*E with a constitutive promoter called p_{repU}. Multiple steps succeeded in execution to properly construct the pMG118 vector, involving the isolation of upstream and downstream gene fragments associated with the P*nprE*, *ylaA*, and *nprE* genes. Initially, the amplified fragments from both genes sustained electrophoresis on a 1.5% agarose gel and subsequently purified. Gel purification treatment used a gel purification kit (Thermo Scientific). These fragments' subsequent cloning into the pGEM-T Easy vector generated pGEM-vector derivatives. The necessary enzymatic digestion and ligation procedures followed the guidelines outlined in the pGEM®-T Easy Vector Systems Technical Manual provided by the Promega Corp. (Madison, WI, USA).

A schematic representation describing the procedure for vector construction appears in Figure 1. Initially, the vector pBG106, containing a cassette composed of p*bp*, P_{repU}-neo, and fenF, underwent double digestion with *Sal*I and *Not*I to obtain the P_{repU}-neo fragment. Subsequently, this fragment's insertion into the pGEM-T Easy prepared pMG112. For the formation of pMG115, double digestion of *ylaA* and pGEM using *Sal*I and *Sac*I, followed by ligation, proceeded.

Table 2. Primer sequences for isolation of partial fragments from *ylaA* and *nprE.*

Name	Primer sequence (5'-3')	Product size (bp)
YlaA fwd	GTCGAC TTATACGTTCGACCTTGCTG	576
YlaA rev	GAGCTC TAAAGTGTTTCATCCGTAGG	
NprE fwd	CCATGGTATCAATCAGCCTGCCAGGT	658
NprE rev	GCATGCAACAGTTGCGCCCTTTAGC	

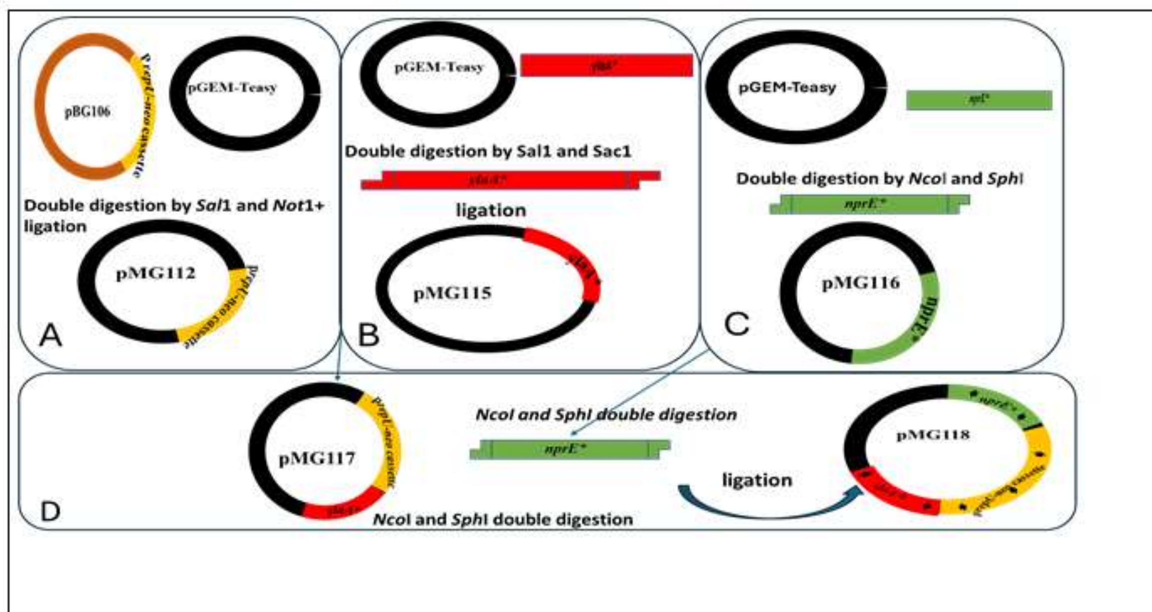

Figure 1. Schematic representation of the construction of recombinant vectors pMG118: A: The construction of recombinant vectors pMG112 by double digestion of pBG106 and pGEM-T by *SalI* and *NotI* and ligation, B: The construction of recombinant vectors, pMG115 containing gene fragment of *ylaA*, C: The construction of recombinant vectors pMG116 exhibiting *nprE* gene fragment, and D: The construction of recombinant vectors pMG118. The arrows on PMG118 vector refer to the direction of genes expression (5' →3').

Similarly, the pMG116 vector generated resulted from inserting the *npr*E fragment into pGEM-T after double digestion with *Nco*I and *Sph*I. Next, the double digestion of pMG115 and pMG112 vectors with *Sal*I and *Sac*I helped insert the *ylaA* fragment into the corresponding sites, forming pMG117. The construction of pMG118 succeeded through a double-digestion process using *Nco*I and *Sph*I enzymes. The same treatment on pMG116 and pMG117 vectors resulted in the release of the *nprE* fragment. The released fragment then became an insertion into pMG117. The constructed vectors' replication occurred within competent *E. coli* DH5α cells, with the plasmid isolation performed using the GeneJET Plasmid Miniprep Kit (Thermo Scientific, USA). Endonuclease digestion continued according to the standard procedures outlined by Sambrook and Russell (2001). Subsequently, the vector pMG118 transformation into competent *BS*168 cells reached plating on LB agar supplemented with neomycin and incubated at 37 °C to select positive transformants.

Enzyme excretion and partial enzyme purification

The wild-type and overproducer mutant strains' initial streaking on LB agar plates was at 37 °C for 18 h. Afterward, a single colony transfer from each strain to 100 ml of LB medium in 500 ml Erlenmeyer flasks reached incubation for 48 h at 37 °C under shaking conditions at precisely 200 rpm. Following this, centrifuging the cultures at 14000 rpm for 10 min collected the supernatant, which served as a source of enzymes. These enzymes attained precipitation using 60%–70% ethanol and quantitatively assayed with Bradford reagent (BioRad, USA). The crude enzyme precipitate surfaced from centrifugation at 14000 rpm for 10 min (Pant *et al.,* 2015). The resulting component constituted the crude extracellular protein extract.

SDS-PAGE electrophoresis

Analyzing the protein content in the crude extracts of both wild and mutant strains employed the Laemmli method (Laemmli, 1970), involving polyacrylamide gel electrophoresis (12% SDS-PAGE). Coomassie Brilliant Blue R-250 and a pre-stained protein ladder (Thermoscientific, USA) treatments helped visually detect the bands and identify the protease molecular weight, respectively.

Nematode biocontrol

The effect of crude extracellular protein extract against *M. incognita* J_2S under in vitro trial on root-knot nematodes, initially isolated from infested fields of the Giza governorate and maintained on tomato roots, served as inoculum in the experiment. Various concentrations of a stock crude enzyme extract began preparation by dissolving 50 mg of precipitated crude enzymes from wild and mutant strains in 5 ml of distilled sterilized water. Their application to a suspension of *M. incognita* J_2S had each milliliter containing 100 ± 3 J_2S. The five established concentrations resulted from dilutions ranging from 1:1 to 1:5 (ml of crude enzyme: ml of distilled sterilized water). One milliliter from each concentration added to one milliliter of J_2S suspension achieved two milliliters. Concurrently, the control group, consisting of distilled water plus boiled crude enzyme with the same concentrations, ran trials under identical conditions. The experiments had five replicates under incubation at room temperature. The nematicidal activity assessment of the crude enzyme extract continued after 24 and 48 hours of incubation through observations under a light microscope.

At the end of the incubation period, the percentage of mortality calculation used the following equation: mortality% = ([C1 - C2] / C1) × 100, where C1 represents the number of live nematode larvae in the control treatment, and C2 represents the number of live J_2S in other trials. Net mortality computation continued by subtracting the nematode recovery in distilled water from the mortality after 48 hours of bacterial treatment (Soliman *et al.,* 2019).

The screen house experiments

A pot experiment commenced in the experimental screen house of the Plant Pathology Department, National Research Centre, Giza, Egypt. The experimental procedures followed those outlined by Darwesh *et al.* (2019); however, the grown plants were three-week-old *Solanum melongena* (CV. Alabaster) eggplant seedlings. The soil drench treatments acquired meticulous application through two methods: the first involved adding enzymes and *M. incognita* J_2S nematodes simultaneously, and the second entailed the addition of crude enzyme a week after nematode infection. Each treatment comprised replications in five sets. After 60 days, the percentage reduction in J_2S, root galls, and egg masses/root systems, versus the control treatment, consisting of distilled water without protease enzymes, succeeded.

Growth and biochemical parameters

Growth parameters

Plant parameters included measurements of the length and fresh weights of shoots and roots in centimeters and grams, respectively. The percentage of plant growth calculated for each criterion underwent comparison with the control treatment (Mohamed *et al.,* 2021).

Biochemical parameters

Biochemical parameters, including polyphenol oxidase, total phenol compounds, β-1,3-glucanase, and chitinase, incur assessment in both treated and control samples. Briefly, using one gram of leaves and roots from each replicate in each treatment served as a source of the biochemicals. Polyphenol oxidase and total phenol compounds measurement followed the method by Vamos-Vigyazo and Nadudvari-Markus (1982). β-1,3-glucanase evaluation was according to the method described by Gupta *et al.* (2012), and garnering chitinase

employed the method according to Rustiguel *et al.* (2012). The extraction of these enzymes utilized the procedure outlined by McCord and Fridovich (1969). Protein content determination engaged the method by Lowry *et al.* (1951), with the bovine serum albumin serving as a standard.

RESULTS

Protease overproduction using site-directed mutation protocol

PCR amplification of ylaA and nprE partial sequences

The sense primer (*ylaA* fwd) and anti-sense primer (*ylaA* rev) generated a fragment of 576 bp corresponding to the *yla*A gene. Another fragment of 658 bp corresponding to *nprE* received amplification by the *nprE* forward and *nprE* reverse primers (Figure 2). Each fragment, cloned separately into the pGEM-T Easy vector, constructed pMG115 and pMG116, respectively. Subsequently, as detailed in the methods section, the final construct of pMG118 succeeded in adequately designing and transforming into the competent cells of *BS*168. In this context, the direction, 5' to 3', of the P_{repU}-*neo* cassette and *npr*E is linear, while that of the *yla*A gene fragment is in the opposite direction. Following the transformation of the competent cells of BS168 with pMG118, positive transformants successfully grew on LB agar media supplemented with neomycin for several generations. Homologous recombination between the constructed vector pMG118 and the *BS*168 chromosome occurred through crossing-over between homologous fragments (Figure 3), causing the replacement of p*nprE* with the constitutive P_{repU}. This replacement subsequently enhanced and increased protease yield. The resulting modified strain attained the designation of *BS*118.

Protease activity and SDS-PAGE electrophoresis

The enzyme-specific activity of the modified strain, *BS*118, rose to 90 U mg-1, which was twice that of the wild-type strain. The enzyme-specific activity of the wild-type strain recorded 43.33 U mg^{-1} in the case of the culture filtrate and 215.4 U mg^{-1} in the crude extracellular protein extract. For *BS*118, the enzyme-specific activity in the crude extracellular protein extract was 101.6 U mg^{-1}. The neutral proteases from *BS*168 and *BS*118 (M3) were named *pro*168 and *pro*118, respectively. After SDS gel electrophoresis, a protein band appeared at a molecular weight of 40 kDa in the crude extracts of *pro*168 and pro118, corresponding to neutral metalloprotease (Figure 4).

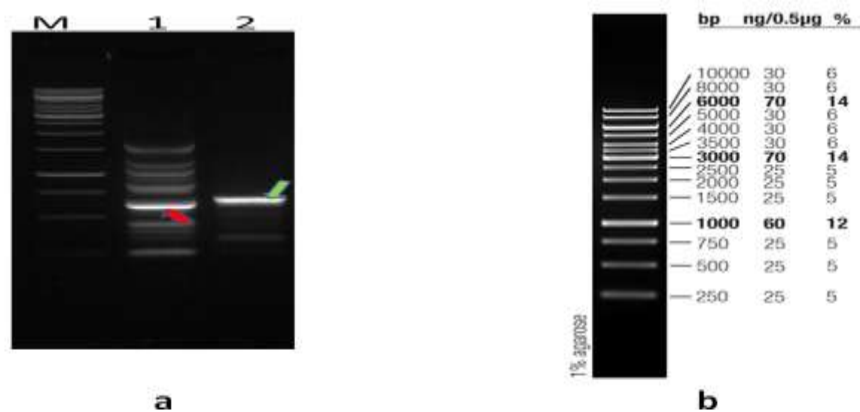

Figure 2. Agarose gel electrophoresis of *ylaA* and *nprE* gene fragments from *Bacillus subtilis* 168. Lane (M): 1kb DNA ladder, Lane (1): *ylaA* was PCR generated at 576 bp, and Lane (2): *nprE* was PCR amplified at 658 bp (a), and Thermo Scientific Gene Ruler 1 kb DNA Ladder (b).

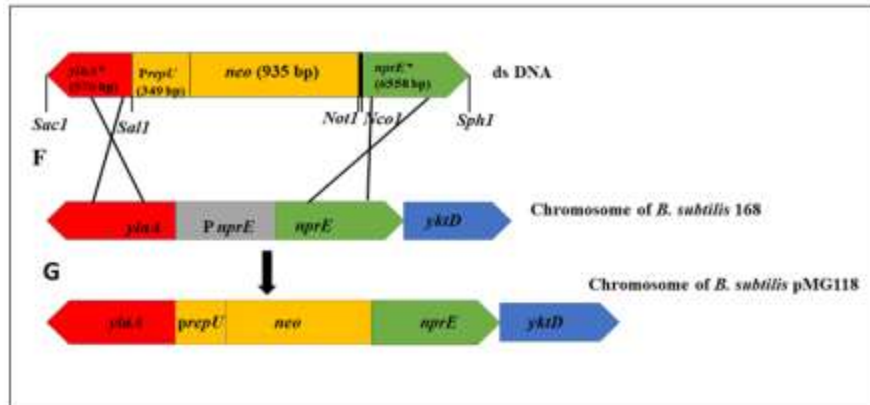

Figure 3. Schematic representation of construction of host strain, *B. subtilis* PMG118. Two DNA fragments of *ylaA* and *nprE* were fused upstream and downstream of P_{repU}-*neo* cassette, respectively, to construct the vector PMG118 (F), and the sequence, P*npE*, between DNA fragments of *ylaA* and *nprE* was replaced by P_{repU}-*neo* cassette via a double-crossover recombination. Crossed lines indicated double-crossing-over and recombination (G).

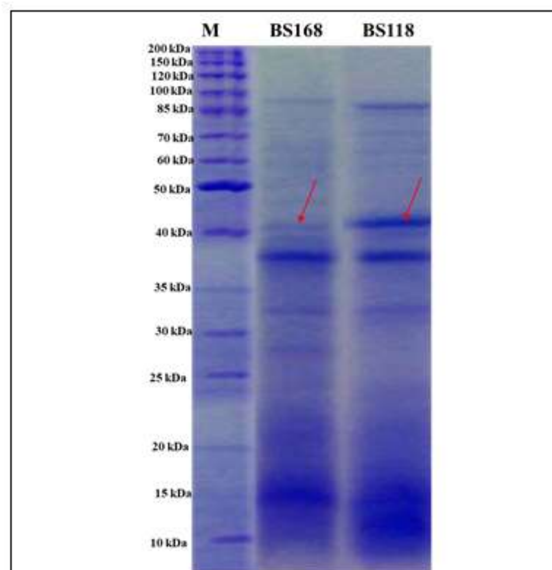

Figure 4. SDS gel electrophoresis of crude extracellular protein extract from *BS*168 and *BS*118. M: protein ladder (Thermoscientific, USA).

Effect of crude protein against M. incognita *J_2S*

Observations revealed that mortality in the case pro118 ranged from 91.3% to 84.5%. The last three concentrations, with dilutions of 1:3 to 1:5, recorded significant mortality rates of 90.1%, 86.27%, and 84.5%, respectively. These rates were more efficient than those of pro168 (Table 3). The morphological differences between live and dead J_2S gained scrutiny under the light microscope according to their movement. The motility, characterized by a winding shape, indicated viability, whereas the absence of movement, represented by a straight shape, provided the mortality of J_2S.

Table 3. The mortality percentage in *M. incognita* J_2S after treatment of different concentrations of pro118 and pro168 crude extracellular protein extract under *in vitro* conditions. The five concentrations were established through dilution ranging from 1:1 to 1:5 (mL of crude enzyme: mL of distilled sterilized water).

Protein treatment	1mL:1mL	1mL:2mL	1mL:3mL	1mL:4mL	1mL:5mL
Pro118	91.3±1.4	91.2±0.9	90.1±1.0	86.27±2.2	84.5±0.8
Pro 168	88.2±1.0	87.3±1.1	43.4±1.6	20.6±0.4	15.5±0.4

Table 4. The reduction in juveniles, root galls, and egg masses number/root systems treated by pro118 crude extracellular protein extract through two applications: the addition of crude enzymes with nematode infection at once and the addition of crude enzymes a week after the nematode infection.

The reduction percentage	At once	After week
J_2S in the soil	88.2±2.4	68.7±1.8
root galls	86.9±1.6	68.1±2.4
egg masses/root	81.5±1.0	55.3±5.2

The screen house experiment

Nematicidal activity of pro118 crude protein

In the screen house experiment, the enzyme pro118 employment was at the initial concentration (1:1). The analysis of this experiment revealed that the treatment involving the concurrent application of the enzyme and nematode yielded greater efficacy. It resulted in a significant reduction of *M. incognita* J_2S in the soil, root galls, and egg masses/root systems by 88.2%, 86.9%, and 81.5%, respectively, compared with the treatment of enzyme addition administered a week following nematode infection (Table 4). Furthermore, all findings reached semblance with the control treatment for comparative assessment.

Effect of pro118 crude protein on growth parameters

Both treatments resulted in a significant ($P \leq 0.05$) increase in plant shoot length and fresh weight compared with the control treatment, following diligent monitoring. The trial involving both enzyme application and nematode infection showed the simultaneous highest increase in the length and fresh weight of the plant shoot. This increase was 9.7% and 14.7% greater, respectively, than the treatment when adding the enzyme a week after nematode infection. Additionally, all findings proceeded with the differentiation with the control treatment for comparative assessment.

Effect of pro118 crude protein on biochemical characters

This experiment aimed to assess specific enzymes involved in the process of plant resistance induction, namely, glucanase (GLU), polyphenol oxidase (PPO), and chitinase (CHI). The data presented in Table 5 demonstrate that both treatments, either enzyme addition to nematode at once or after a week, caused an increase in these enzymes in plant leaves compared with the control treatment. Notably, the first treatment showed the highest activity increase for polyphenol oxidase and glucanase, recording 6.3 and 6.1 U mg^{-1}, respectively, compared to 3.3 and 0.7 U mg^{-1} in the second treatment. However, no significant difference occurred in chitinase activity in any treatments. The increase in the specific activity of total phenol compounds was approximately the same in both treatments, with the particular activity of TPC recorded at 0.4 U mg^{-1} protein in the control treatment.

Table 5. Estimation of Polyphenol oxidase, β - 3,1, Glucanase, Chitinase, and Total Phenol compounds specific activities (u mg^{-1} protein) in eggplant leaves treated by *pro*118 crude extracellular protein extract through two applications: addition of crude enzymes with nematode infection at once and addition of crude enzymes a week after the nematode infection. Control: addition of heat killed crude extracellular protein extract dissolved in sterilized water.

Treatments	Polyphenol oxidase ($U\ mg^{-1}$ protein)	Glucanase ($U\ mg^{-1}$ protein)	Chitinase ($U\ mg^{-1}$ protein)	Total Phenol compounds (mg/g)
at once	6.3± 0.54	6.1± 0.75	0.34± 0.004	6.6± 0.53
after week	3.3±0.32	0.7± 0.31	0.33± 0.007	6.3± 0.57
control	0.0	1.0± 0.009	0.31± 0.006	0.4± 0.11

DISCUSSION

The beneficial role of microbial protease as a nematicidal bioagent has many researchers proving it. Darwesh *et al*. (2019) reported the efficiency of the thermostable alkaline protease secreted by *Saccharomonospora viridis* in controlling harmful root-knot nematodes. Li *et al*. (2023) stated that the nematicidal activity of crude extracellular protein extract was more effective than the culture filtrate from *B. cereus* NJSZ-13 against pinewood nematodes. However, few reports have investigated the role of metalloprotease from *Bacillus spp.* on species of nematodes other than *M. incognita* juveniles (J_2S). In this study, the enhancement of protease production originating from *BS*168 succeeded by site-directed mutation. This approach replaced the native promoter that regulates the expression of neutral protease with the constitutive promoter P_{repU}. Previous studies have shown the efficiency of this promoter in enhancing some secondary metabolites. Leclère *et al*. (2005) replaced the native promoter of the mycosubtilin operon with P_{repU}, resulting in a 15-fold increase in the yield of mycosubtilin compared to the wild type, *B. subtilis* ATCC 6633.

Hussein and Fahim (2016) created a mutant strain, BMG06, that overexpressed plipastatin and fengycin by about 35 and four folds, respectively, more than the wild-type *B. subtilis* 168 by promoter replacement. The strain of *Bacillus subtilis* encodes eight extracellular proteases, consisting of five serine proteases and three metalloproteases (*Apr*E, *Npr*E, and *NprB*). Among these, the most abundant is the neutral metalloprotease (NMP) encoded by *NprE*, which accounts for over 95% of *B. subtilis* extracellular proteolytic activity (Dalamu *et al*., 2012; Barbieri *et al*., 2016; Parnidi *et al*., 2021). The observed increase in NMP activity was only twice as much as that of the wild type, possibly because the NMP enzyme secreted in large quantities already occurred. Consequently, exceeding the permissible limit in enzyme synthesis may be toxic to the cells, depending on the genetic behavior of the host system.

Given the concerns about the environmental release of transgenic bacteria, the study further enhanced and employed the crude extract of the protease, *pro*118, as a pathogenic agent against root-knot nematodes (RKN). Initially, in the in vitro experiment, no discernible morphological differences appeared in dead juveniles' motility and viability after treatments with either *pro*168 or *pro*118 crude enzyme extracts. The only distinction was in the mortality percentage, with *pro*118 surpassing *pro*168 due to its higher concentration. If we hypothesize the presence of other toxic peptides besides protease in the crude extracellular protein extract, it can be possible that these peptides demonstrated a nematicidal effect. However, the higher nematicidal concentration of pro118 than that of *pro*168 was often because of its higher concentration. This is primarily because the crude extract of enzymes in both *BS*168 and *BS*118 contained the same concentration of other peptides. Study findings exhibited some inconsistency with the results obtained by Niu *et al*. (2006) and Lian *et al*. (2007), who demonstrated that extracellular neutral metalloproteases from *B. subtilis* exhibited minimal nematicidal activity in vitro and could not degrade the intact cuticle of the free-living

nematode *Panagrellus redivivus*. Noteworthily, in this study, both *pro*118 and *pro*168 proved toxic against *M. incognita* J_2S when applied at higher concentrations. These findings aligned with studies mentioning that the cuticle of the dead nematode remained intact and showed no apparent damage. However, the nematodes were immobilized and lacked movement even after water recovery. It suggests that the neutral protease could degrade proteins from the inner layer of purified cuticles from the nematode, making the cuticle appear intact. In contrast, the serine alkaline protease could hydrolyze the protein matrix embedded in the nematode's cuticles (Li *et al.,* 2023).

In the screen house experiment, the most effective treatment involved the simultaneous application of crude enzymes with nematode infection. This efficacy can refer to the early prevention of nematodes from penetrating and prospering into the root system under the influence of the protease extract. On the other hand, the second treatment, involving the enzyme addition one week after nematode infection, resulted in lower mortality. The reason may be that the nematodes had likely already penetrated the roots, establishing an indirect connection with the enzyme. It differs from the application of bacterial cells, where the latter can interact directly and indirectly with plants and their pathogens, such as root-knot nematodes (RKN) (Mohamed *et al.,* 2021; Darwesh *et al.*, 2019). The increased growth parameters observed in eggplants can be because releasing protease was during the infection process. Consequently, nutrients such as amino acids and small peptides improve the growth of host microorganisms, promoting the plant's immune system to reduce nematode infections and enhance growth parameters, including increased root and shoot weight and length (Li *et al*., 2023).

Interestingly, bacteria with plant growth-promoting properties (PGPB) are crucial in improving plant health and inducing resistance mechanisms. They do this by overproducing plant defense hormones and enzymes, such as catalase, chitinase, peroxidase, phenylalanine ammonia-lyase, and polyphenol oxidase (Aquino *et al.,* 2019; Tiepo *et al.,* 2020). The pooled data from the screen house experiment showed a boost in the production of polyphenol oxidase, total phenol compounds, and β-1,3-glucanase. These findings support the vital roles of these biochemicals in enhancing plant health. Study results confirm the findings of Khanna *et al*. (2019), who reported that applying organic fertilizers and bio-agents increased the activities of defense-related enzymes, such as peroxidase (POX), polyphenol oxidase (PPO), and phenylalanine ammonia oxidase. It led to the oxidation of total phenol compounds (TPC) to quinones, which are more toxic than the original phenols. This process often induces resistance against plant pathogens (Mayer, 2006).

Furthermore, the improved nematicidal effect can refer to the increase in glucanase (GLU), as it can degrade the cell walls of pathogenic agents and hydrolyze corresponding substrates (Zinov'eva *et al.,* 2001). However, these results contrasted with the findings of Abd-Elgawad and Kabeil (2012), who reported that nematode infection had no effect on GLU activity but increased PPO activity in inoculated roots compared with uninoculated ones. In conclusion, the combined data collected from the screen house experiment corroborated the findings derived from the in vitro trial when applied as a one-time soil drench. Enhanced effectiveness could potentially be successful with the application of these enzymes in additional doses.

CONCLUSIONS

Using biological agents instead of chemical nematicides shows high potential for controlling different plant infections, like root-knot nematodes (*Meloidogyne spp*.). By genetically engineering techniques to increase microbial protease production, specifically by replacing the native promoter with a constitutive one, the suppression of *M. incognita* and improvement of plant parameters surged. This approach is probably

advantageous in creating strongly modified strains as bioagents, and further research is necessary to enhance their effectiveness in practical applications.

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