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Castanea crenata Ginkbilobin-2-like Recombinant Protein Reveals Potential as an Antimicrobial against *Phytophthora cinnamomi*, the Causal Agent of Ink Disease in European Chestnut

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Abstract: The European chestnut tree (*Castanea sativa* Mill.) is widely cultivated throughout the world's temperate regions. In the Mediterranean region, it has a significant economic role mainly because of the high quality of its edible nuts. The Oomycete *Phytophthora cinnamomi* is one of the most severe pathogens affecting European chestnuts, causing ink disease and significant losses in production. Ginkgobilobin-2 (Gnk2) in *Ginkgo biloba* is a secreted protein with a plant-specific cysteine-rich motif that functions as a lectin, and its carbohydrate-binding properties are closely related to its antifungal activity. The binding of lectins to mannose residues of the cell wall of *Phytophthora* species may disturb and disrup the cell wall structure. This work determined that the amino acid sequence has a signal peptide that directs the final protein peptide to the apoplast. The Cast_Gnk2-like expression was performed and optimized, and different in vitro antagonism tests were done against *P. cinnamomi* using different purified protein concentrations. As a result of one of these assays, Cast_Gnk2-like significantly reduced the mycelia growth of *P. cinnamomi* in liquid medium as shown by the mycelia weight (g) in control treatments was 377% higher than in the treatments. These insights reveal the potential of Cast_Gnk2-like for agricultural uses and biotechnological developments for the pathosystem chestnut/*P. cinnamomi*.

Keywords: Antagonisms; *Castanea sativa*; in vitro biotic interactions; pathosystem chestnut/*P. cinnamomi*; Phytophthora cinnamomi

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Citation: Colavolpe, M.B.; Vaz Dias, F.; Serrazina, S.; Malhó, R.; Lourenço Costa, R. *Castanea crenata* Ginkbilobin-2-like Recombinant Protein Reveals Potential as an Antimicrobial against *Phytophthora cinnamomi*, the Causal Agent of Ink Disease in European Chestnut. *Forests* **2023**, *14*, 785. https://doi.org/10.3390/f14040785

Academic Editors: Ricardo Javier Ordás and José Manuel Álvarez Díaz

Received: 26 February 2023
Revised: 30 March 2023
Accepted: 5 April 2023
Published: 11 April 2023

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1. Introduction

The genus *Castanea* belongs to the Fagaceae family, which dominates much of the hardwood forests of the Northern Hemisphere. The European chestnut (*Castanea sativa* Mill.) is considered to be the only native chestnut species in Europe. This species is a multipurpose tree, that is used in the food industry for its high-quality edible fruits (nuts), in the forest industry for timber, and also for ecological and landscaping purposes [1].

Over 100 species have been described within the genus *Phytophthora* [2]. According to many authors [3,4], most survive in the soil for long periods without a host and affect the parts of the plant in contact with the ground, thus causing a destruction of the root system. The impact of this group of organisms is mirrored in its name, as *Phytophthora*, which means, in Greek, plant destroyer [1,4].

The rural economy of the northern mountainous regions of Portugal are mainly based on chestnut culture. Unfortunately, the current area and productivity per hectare are

much less than the country's potential, mainly due to root rot caused by *Phytophthora cinnamomi* Rands, the main threat to chestnut orchards in Europe [5]. *Castanea sativa* was severely affected and nut production has declined by 251,549 tons from 1961 to 2015 [1,6]. The economy of mountainous areas in the Mediterranean regions of southwest Europe mainly depends on the significant income that chestnut culture represents to the local farmers, which is sometimes the only income they can get from the land. In recent years, the persistent losses of chestnut trees to *P. cinnamomi* is contributing to a decline in the population of these regions [6].

Phytophthora cinnamomi infects the roots and after reaching the vascular tissues it continues to colonize the roots until it obstructs the xylem vessel, thus restricting root growth and interfering with water and nutrient uptake to the plant shoot. The roots and root collar start to rot, resulting in a progressive decline of the tree. The above-ground symptoms include chlorosis and wilting of leaves, dieback of branches, defoliation, and gradual decline until the tree dies [1]. *Phytophthora cinnamomi,* has an extensive host range, destroying thousands of plant species worldwide and causing devastating impacts on natural ecosystems, agriculture, and forestry [6].

The coexistence of Asian chestnut species from Japan and China (*Castanea crenata* Sieb. & Zucc. and *Castanea mollissima* Blume) with *P. cinnamomi* (originally from the Asian tropics) lead to the development of resistance against the pathogen during the evolution process. The secretion of antifungal proteins and cell wall reinforcement are part of the constitutive defense barriers to pathogen growth in the plants that may explain the difference in resistance to *P. cinnamomi* between *C. crenata* and *C. sativa.* Although the Asian species have proven resistant to ink disease, these species are not adapted to the Atlantic environmental conditions. Therefore, the resistance of Japanese and Chinese chestnut species to this pathogen led to their introduction in breeding programs over the last years as donors of resistance in controlled crosses [7].

Over time, several approaches have been proposed to deal with root diseases (which affect the xylem of trees), such as the intensive application of pesticides and the selection and production of improved plant material with some disease resistance [8]. Phosphite and metalaxyl have been the most frequently used chemicals against *P. cinnamomi.* Nevertheless, the continuous use of these two chemicals has led to the development of tolerance within the pathogen [1]. Effective biological control methods have not been developed to date [1]. More common control approaches involve using resistant rootstocks for propagation or planting of resistant hybrids [1]. Nevertheless, the measures and procedures have not yet been successful, mainly due to the easy development and migration of *P. cinnamomi* zoospores in wet conditions, especially during rainfalls and waterlogging and because of the resistance structures that persist in the soil and are extremely difficult to eradicate [1,9].

A previous study identified a secreted protein with antifungal activity, Ginkbilobin2 (Gnk2), from seeds of the gymnosperm *Ginkgo biloba* [10]. Gnk2 comprises 108 amino acids as a mature protein with a plant-specific cysteine-rich motif. It functions as a lectin [3,11], and its carbohydrate-binding properties are tightly related to its antifungal activity. It binds with high affinity to D-mannose and with less affinity to D-glucose [11], and both exist in the hyphal cell walls of *Phytophthora* species [12].

Our group has been studying the *C. sativa* and *C. crenata* root transcriptome in response to *P. cinnamomi* to elucidate chestnut defense mechanisms. In a previous study [13], we identified candidate genes differentially expressed in the roots of the susceptible species to the pathogen (*C. sativa*), and the resistant one (*C. crenata*) observed after *P. cinnamomi* inoculation. The research demonstrated that both species recognize the pathogen attack, but only the resistant species (*C. crenata*) may involve more genes in the defense response than the susceptible species (*C. sativa*). RNA-seq analysis further enabled the selection of candidate genes for ink-disease resistance in *Castanea* [12]. Following the same rationale to elucidate chestnut defense mechanisms against *P. cinnamomi,* our group selected the Ginkgobilobin-2-like gene (*Cast_Gnk2-like*) from the transcriptomes of *C. crenata*, intending to evaluate the early expression of candidate resistance genes in both species (*C. sativa* and

C. crenata). *Cast_Gnk2-like* was the most expressed gene and the one that best discriminates between susceptible and resistant chestnut genotypes. The highest *Cast_Gnk2-like* expression registered in non-inoculation conditions suggests that *C. crenata* root surroundings may be a hostile environment for fungal and fungal-like pathogens, such as *P. cinnamomi*. On the other hand, *C. sativa* showed a very low *Cast_Gnk2-like* expression level, even after pathogen inoculation [7].

The present work is part of an ongoing research program that aims to find solutions to this important problem regarding the Chestnut/*P. cinnamomi* pathosystem in Europe. It was reported that Gnk2 inhibits the growth of pathogenic fungi such as *Fusarium oxysporum*, *F. culmorum*, and *Candida albicans*; and activates actin-dependent by inducing hypersensitive response (HR-related) plant cell death [10]. The homology of *Cast_Gnk2-like* with *Gnk2* led us to test the encoded protein against *P. cinnamomi*. The present work aims to express and produce the *Cast_Gnk2-like* protein for evaluation of its effectiveness on in vitro assays as a possible antagonist of the *P. cinnamomi* pathogen.

2. Materials and Methods

2.1. Cast_GNK2-like In Silico Analysis

The nucleotide sequence of the C. crenata *Cast_GNK2-like* transcript was obtained from the sequenced root transcriptome after *P. cinnamomi* inoculation [13]. It was annotated as a Cysteine-rich repeat secretory protein 38. After a BLASTn at NCBI and a comparison of the sequences with the highest homology, a prediction of the coding sequence and translation to the amino acid sequence was achieved. A BLAST to *Cast_GNK2-like* was achieved in Uniprot [14] to identify the proteins with higher similarity. Uniprot was also used to predict the protein families and domains of *Cast_GNK2-like* by comparison with the ones of *Ginkgo biloba* (GNK2), *Arabidopsis thaliana*, and *C. mollissima*. Signal peptides for *Cast_GNK2-like* were searched using SignalP—6.0 [15] and TargetP—2.0 [16]. Alignment of the amino acid sequences of *Castanea mollissima* Cysteine-rich repeat secretory protein 38 (A0A8J4V9V8), *Ginkgo biloba* Antifungal protein ginkbilobin-2, C. crenata putative Ginkbilobin-2 protein and *A. thaliana* Putative cysteine-rich receptor-like protein kinase 9, was performed using CLUSTALW [17].

2.2. Bacterial Strains and Growth Condition

Escherichia coli bacteria strains used in this study were TOP10 and BL21 (DE3) pLysS (competent cells from Thermo Fisher Scientific Inc., Waltham, MA, USA). *E. coli* strains containing recombinant plasmids were cultured, at 37 °C, in LB broth medium supplemented with 100 μg/mL ampicillin or 50 μg/mL kanamycin.

2.3. Phytophthora Cinnamomi Strain

Phytophthora cinnamomi (strain PH107) was isolated at the UTAD (the University of Trás-os-Montes and Alto Douro), from Vila Real (Portugal), and it is preserved at the INIAV I.P. (Instituto Nacional de Investigação Agrária e Veterinária I.P.) in Lisbon. It is routinely cultured at 25 °C on Potato-Dextrose Agar (PDA) in Petri plates and maintained in darkness. For all the assays, a fresh culture of *P. cinnamomi* was prepared by transferring a small piece of mycelium from a previous culture to a new plate of PDA nutritive medium and incubated in darkness at 25 °C for 4/5 days (or maximum one week).

2.4. Plasmid Construction

The designed primers for the amplification of the *Cast_Gnk2-like* coding sequence were 5′–CTCCATATGgctgacccattataccatttttgttttag–3′ and 5′– CACGGATCCctaggcatcaacaaag -gggta–3′. The PCR was performed using Phusion™ High-Fidelity DNA Polymerase (2 U/μL) according to the manufacturer protocol. The vectors used for the construction were pET15b and pET28a (Novagen®, Gujarat, India), which carry an N-terminal or an N- and C- terminal oligo-histidine tag (6x His-tags), respectively. The DNA sequence encoding *Cast_Gnk2-like* was inserted into the vectors between *Nde*I and *Bam*HI sites.

Control plasmids were constructed without the *Cast_Gnk2-like* fragment. It is expected to observe a protein fraction (a band in the gels) in the region of 25 kDa. The size of the *Cast_Gnk2-like* coding sequence is represented in Figure 1 (726 bp).

```
1     atg ttg agc tca aaa tat att tct gtc agc ttt cta tta ctc agc   45
1      M   L   S   S   K   Y   I   S   V   S   F   L   L   L   S   15

46    ctc tcc ctc cat gca gtc aat tgt gct gac cca tta tac cat ttt   90
16     L   S   L   H   A   V   N   C   A   D   P   L   Y   H   F   30

91    tgt ttt agc caa gaa agc tac act gcc act agc cgt tat ggt aca   135
31     C   F   S   Q   E   S   Y   T   A   T   S   R   Y   G   T   45

136   aac ttg aat ggc ttg ctc aat ctt ttg tcc acc aaa gtt cct tca   180
46     N   L   N   G   L   L   N   L   L   S   T   K   V   P   S   60

181   aaa ggg ttt ggt ctc agc tcg act ggg caa ggc caa gat cga gca   225
61     K   G   F   G   L   S   S   T   G   Q   G   Q   D   R   A   75

226   aat ggt tta gcc cta tgc cgg ggt gat gtc tca aaa aca aac tgt   270
76     N   G   L   A   L   C   R   G   D   V   S   K   T   N   C   90

271   acg acc tgt gtc att gat gca ggc aaa gag ctt ggt aat cgt tgt   315
91     T   T   C   V   I   D   A   G   K   E   L   G   N   R   C   105

316   cct tat aaa aaa gga gcg ata att tgg tat gat aac tgt ctt ttg   360
106    P   Y   K   K   G   A   I   I   W   Y   D   N   C   L   L   120

361   aag tac tcg aac att gat ttc ttt gga gaa atc gat aac aaa aac   405
121    K   Y   S   N   I   D   F   F   G   E   I   D   N   K   N   135

406   aag ttc tac atg tgg aac gtc caa gat gta gaa aat ccc act tca   450
136    K   F   Y   M   W   N   V   Q   D   V   E   N   P   T   S   150

451   ttc aat cca aaa gtt aag gat ttg tta agc agg tta tct aat aaa   495
151    F   N   P   K   V   K   D   L   L   S   R   L   S   N   K   165

496   gct tat gcc aat cca aaa ttc tat gct acc ggg gac cta aag ctt   540
166    A   Y   A   N   P   K   F   Y   A   T   G   D   L   K   L   180

541   gac tca tca agc aaa cta tat ggt ttg gct caa tgc acc agg gac   585
181    D   S   S   S   K   L   Y   G   L   A   Q   C   T   R   D   195

586   cta tca ggt ctt gat tgt aag aag tgt ctt gat act gcg att agt   630
196    L   S   G   L   D   C   K   K   C   L   D   T   A   I   S   210

631   gaa ctt ccc aac tgt tgc gat gga aaa cga ggt ggg cga gtt gtt   675
211    E   L   P   N   C   C   D   G   K   R   G   G   R   V   V   225

676   ggt ggc agt tgt aac gtt aga tat gaa ctt tac ccc ttt gtt gat   720
226    G   G   S   C   N   V   R   Y   E   L   Y   P   F   V   D   240

721   gcc tag   726
241    A   *
```

Figure 1. Sequences S1. Cast_GNK2-like coding sequence (up) and respective amino acid sequence (below). In red is the prediction of the signal peptide to the apoplast. In blue, the two conserved cysteine motifs attributed to the antimicrobial action. *: stop codon.

2.5. Protein Expression

2.5.1. Preparation of Bacterial Cellular Lysates

The cell culture was centrifuged at 4000 g for 20 min, and the pellet was resuspended in a 50 mM sodium HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.5, containing 1 M NaCl and 10 mM imidazole (1,3-diazaciclopenta-2,4-dieno). Cells were disrupted by sonication. The cell debris was removed by centrifugation, and the supernatant was filtrated (0.45 μm) before the protein purification process.

2.5.2. Protein Purification and Electrophoresis (SDS-PAGE)

The supernatant (cell-free extract) obtained after centrifugation was applied to 5 mL Ni^{2+} ion chelating immobilised ion affinity chromatography column (HiTrap, GE Healthcare, Chicago, IL, USA). The column was preequilibrated with 50 mM sodium HEPES buffer, pH 7.5, containing 1 M NaCl and 10 mM imidazole. The recombinant protein, Cast_Gnk2-like, was eluted with a linear gradient of imidazole (0–300 mM) in 50 mM sodium HEPES buffer, pH 7.5, containing 1 M NaCl. Protein purity was evaluated by SDS–PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), performed in a Protean II xi cell (Bio-Rad, Hercules, CA, USA) at 480 V and 120 mA. The running buffer contained 25 mM Tris base, 0.2 M glycine, and 0.1% SDS. Gels were stained with Coomassie (Bio-Rad Biosafe, Hercules, CA, USA). The protein concentrations were determined using Bradford reagent (using bright blue Coomassie G-250) from BioRad utilizing bovine serum albumin (BSA) as the standard.

2.6. Western Blot

Identification of antigens recognized by serum antibodies was carried out after the separation of total antigens by SDS-PAGE and Western blotting [18]. Following SDS-PAGE, the separated proteins were transferred to nitrocellulose sheets (Amersham™ Protran® Premium 0.2 µm pore size NC). The sheets were blocked with phosphate-buffered saline, pH 7.2, containing 0.3% Tween-20 (0.3% PBS-T), and washed twice. Then they were incubated with His-Tag Antibody (AD1.1.10) solution for one hour at room temperature, always in soft shaking. After washing them three times with 0.3% PBS-T, the secondary antibody solution (m-IgGκ BP-HRP) was added for one hour in the same conditions. After that, another three washes with 0.3% PBS-T were done.

Immunoreactive spots were detected with a chemiluminescence-based kit (Amersham Pharmacia Biotech, Amersham, UK). Before immunodetection, the nitrocellulose membranes were stained with 0.2% *w*/*v* Ponceau S in 3% *w*/*v* trichloroacetic acid for 3 min.

2.7. Cast_Gnk2-like In Vitro Antagonist Activity against P. cinnamomi

Two types of antagonist assays were performed. One of them was done in 60 mm diameter Petri dishes to evaluate *P. cinnamomi* mycelium development with different Cast_Gnk2-like pure protein concentrations (0.2 and 0.5 mg/mL). Briefly, in the solid medium assay, mycelial discs of 5-mm diameter were taken from the margins of approx. 1-week-old *P. cinnamomi* cultures and placed on new PDA medium plates. Different protein concentrations were tested and first spread in all the plates homogeneously, forming a thin film. The Petri plates were left for 25 min inside the flow chamber to dry the liquid above the solid medium before inoculation with *P. cinnamomi*. Different volumes of pure protein were also tested (100, 250, and 500 µL/plate). Control PDA medium Petri plates contained buffer solution without Cast_Gnk2-like protein. The plates were kept at 25 °C in darkness, and the growth of *P. cinnamomi* was evaluated during the following days of incubation (mainly 24, 48, and 72 h). Photographs of the mycelial growth were taken and the area determined with Image J software (Version 1.51r, NIH, Rockville, MD, USA). The second assay was performed in a liquid culture using Potato-Dextrose medium (50 mL) in Erlenmeyer flasks. Mycelial discs (10 discs of 5-mm diameter) were added to each flask and growth of *P. cinnamomi* was evaluated by determining mycelial dry weights (g) after two days of shaking (180 rpm) at 25°C. Controls were buffer solution without Cast_Gnk2-like protein.

2.8. Statistics

For fungal inoculation experiments, at least 10 biological replicates were used for each test, and the experiments were performed 3 times with similar results. The results shown in the pictures are examples from representative samples of *n* repetitions. The area of *P. cinnamomi* mycelia growth expressed in mm^2 was determined. Graph Path software was used to prepare the figures, and the results are shown as means ± SEM. Data were analyzed

by appropriate *t*-test or ANOVA followed by post hoc comparisons by Tukey or Dunnett's test.

3. Results

3.1. Cast_GNK2-like Predictably Has Two Sites for Microbial Binding

Ginkbilobin-2 protein from G. biloba (GNK2) was the most thoroughly characterized so far. It has one conserved cysteine motif (C-8X-C-2X-C) related to its antimicrobial action [8]. We detected two cysteine motifs in Cast_GNK2-like (Figure 1). The same occurs with A. thaliana CRK9 and C. mollissima A0A8J4V9V8 (Table 1, Pfam column). As expected, the protein with the highest similarity to Cast_GNK2-like is A0A8J4V9V8 (99.6%, E-value 3.3×10^{-180}) from the Chinese chestnut. Additionally, the amino acid sequences of all the proteins start with a signal peptide that guides the final peptide to the apoplast (Table 1). The alignment of the four amino acid sequences also indicates that all the proteins have a signal peptide for the protein to be secreted. Moreover, the alignment shows the existence of one conserved cysteine motif for GNK2 and two motifs for Cast_GNK2-like, as A0A8J4V9V8 and CRK9 (Figure 2).

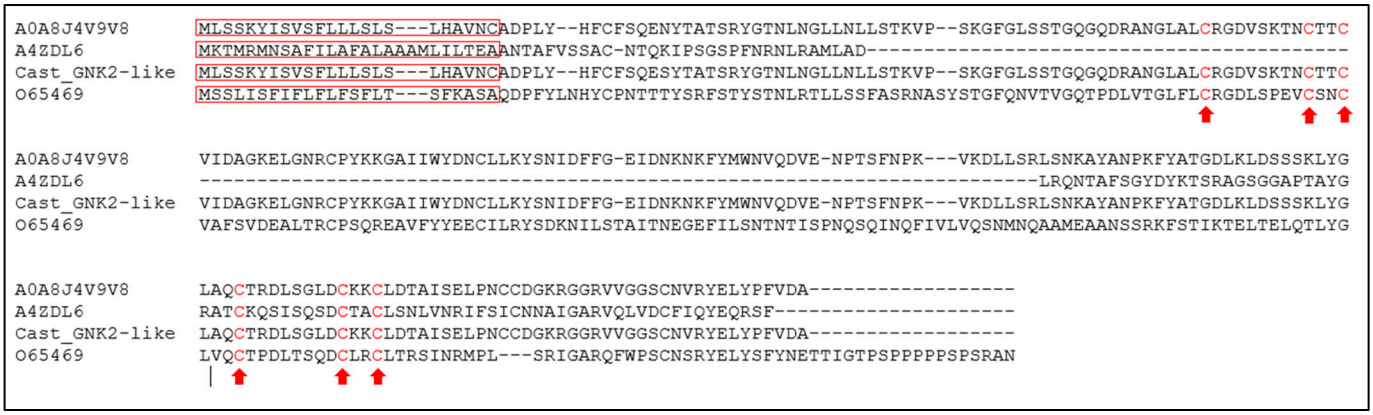


Figure 2. A multiple alignment of *C. crenata* putative Ginkbilobin-2 protein (Cast_GNK2-like), *C. mollissima* Cysteine-rich repeat secretory protein 38 (A0A8J4V9V8), *Ginkgo biloba* Antifungal protein ginkbilobin-2 (A4ZDL6), and *A. thaliana* Putative cysteine-rich receptor-like protein kinase 9 (O65469), with CLUSTALW [17]. Protein accession numbers are indicated (Uniprot Accession). The signal peptide is surrounded by a red box and the conserved cysteine motif (C-8X-C-2X-C) is indicated in red.

Table 1. In silico characteristics of the amino acid sequences homologous to Ginkbilobin-2. N.d.: non-determined. Pos: position of the signal peptide.

Species	Protein Name (Uniprot)	Gene Acronym	Uniprot Accession	Length (AA)	Interpro Family/Domain	Pfam Family, #Hits	Subcellular Localization (Uniprot)	SignalP	TargetP
Ginkgo biloba	Antifungal protein ginkbilobin-2	GNK2	A4ZDL6	134	Gnk2-homologous domain (IPR002902); Gnk2-homologous domain superfamily (IPR038408)	Salt stress response/antifungal (PF01657), 1	Secreted	Secretory	Pos 1–26/27
Arabidopsis thaliana	Putative cysteine-rich receptor-like protein kinase 9	CRK9	O65469	265	Gnk2-homologous domain (IPR002902); Gnk2-homologous domain superfamily (IPR038408)	Salt stress response/antifungal (PF01657), 2	Secreted	Secretory	Pos 1–23/24

Table 1. *Cont.*

Species	Protein Name (Uniprot)	Gene Acronym	Uniprot Accession	Length (AA)	Interpro Family/Domain	Pfam Family, #Hits	Subcellular Localization (Uniprot)	SignalP	TargetP
Castanea mollissima	Cysteine-rich repeat secretory protein 38	N.d.	A0A8J4V9V8	241	Gnk2-homologous domain (IPR002902); Gnk2-homologous domain superfamily (IPR038408)	Salt stress response/antifungal (PF01657), 2	N.d.	Secretory	Pos 1–23/24
Castanea crenata	N.d.	Cast_GNK2-like	N.d.	241	N.d.	N.d.	N.d.	Secretory	Pos 1–23/24

3.2. Protein Isolation and Purification

The results of the recombinant protein expression, following different conditions to optimize the process, showed (according to the electrophoresis gels, SDS-PAGE) that the best expression was obtained using the *E. coli* BL21 (DE3) pLysS, in plasmid pET15, for 16 h at 20 °C, using 1 mM IPTG. We could observe a band in the gels (a protein fraction) in the region of 25 kDa (Figure 3A).

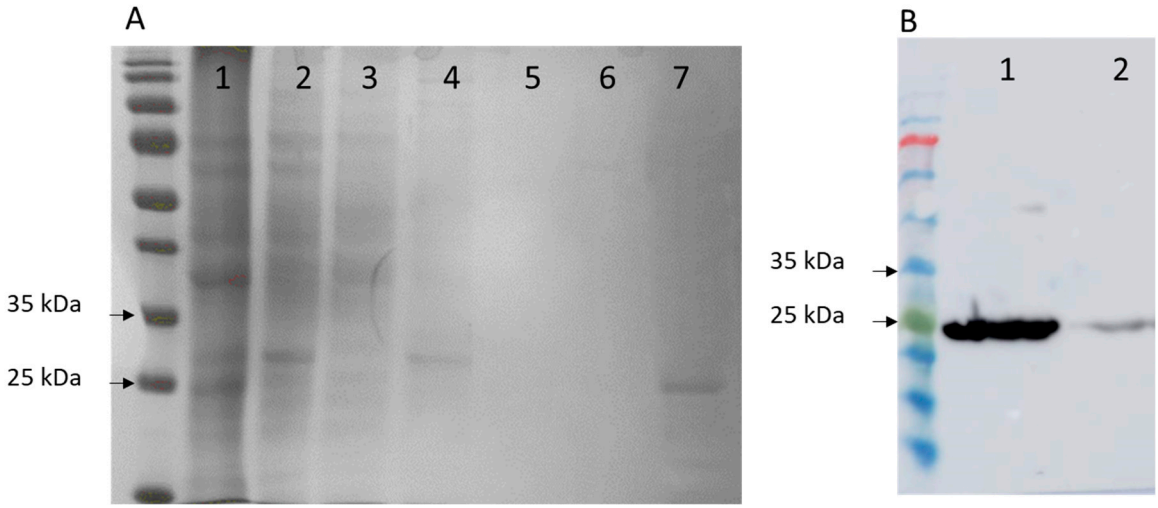


Figure 3. SDS-PAGE gel (14%), panel A, and Western blotting showing the immunodetection of Cast_Gnk2-like protein, panel B. Hyper-expression and purification of Cast_Gnk2-like protein obtained using the E. coli BL21 (DE3) pLysS transformed with pET15b_Cast_Gnk2, lane 1: Cell pellet after sonication; lane 2: cell free extract; lane 3 to 6: fractions of the column washing procedure wash; lane 7: purified recombinant enzyme, 0.5 mg/mL (**A**). Western blotting showing the immunodetection of Cast_Gnk2-like protein using total cell extract after sonication, lane 1—insoluble fraction, lane 2—soluble fraction (**B**). The molecular masses (kDa) of protein standards (NZYTech Ltd., Lisbon, Portugal) are indicated.

The immunoreactive spots in the region of 25 kDa from the Western Blot were detected with a chemiluminescence-based kit (Figure 3B).

3.3. Cast_Gnk2-like In Vitro Activity against P. cinnamomi

Mycelial growth of *P. cinnamomi* in PDA medium containing 0.5 mg/mL Cast_Gnk2-like pure protein was significantly less than the control treatment (without the protein). Figure 4 shows the growth of *P. cinnamomi* mycelia with and without Cast_Gnk2-like pure protein after 24 and 48 h at 25 °C in darkness. Cast_Gnk2-like does not inhibit the growth of *P. cinnamomi*, but it significantly reduces and delays its development.

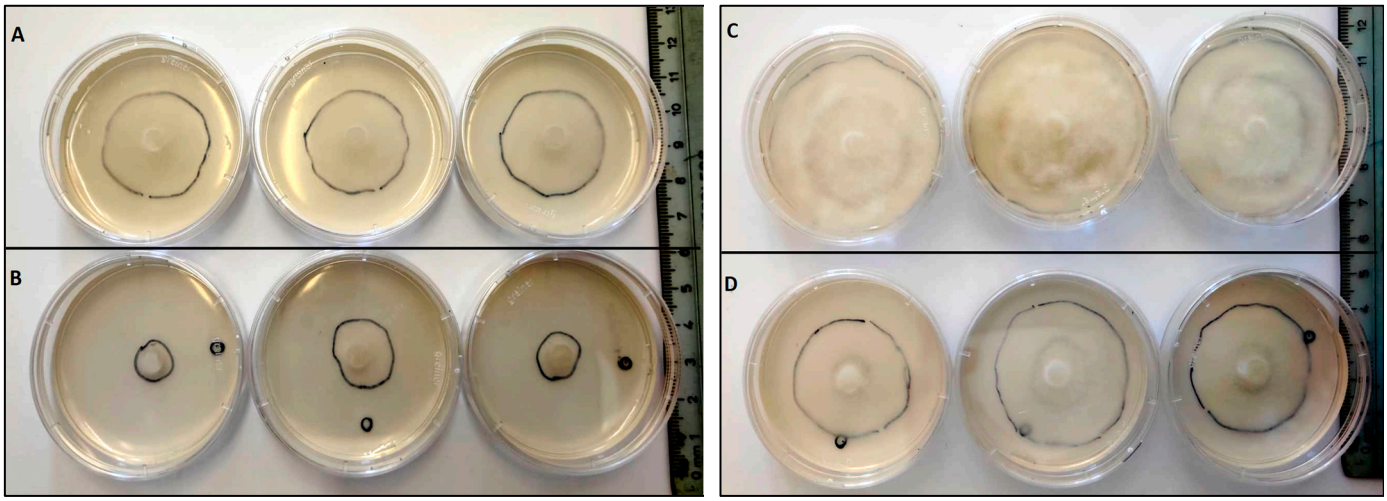


Figure 4. Mycelia of *P. cinnamomi* growth in PDA medium under different conditions (3 Petri plates out of *n* are shown as representative of the assay). (**A**): Control treatment with buffer solution and *P. cinnamomi* mycelial growth after 24 h. (**B**): Cast_Gnk2-like pure protein (250 µL/plate and 0.5 mg/mL) and *P. cinnamomi* mycelial growth after 24 h. (**C**): Control treatment with buffer solution and *P. cinnamomi* mycelial growth after 48 h. (**D**): Cast_Gnk2-like pure protein (250 µL/plate and 0.5 mg/mL) and *P. cinnamomi* mycelial growth after 48 h.

The mycelia area (cm^2) of *P. cinnamomi* growth in PDA medium under control treatment was 7.8 cm^2, while in Cast_Gnk2-like pure protein treatment (0.5 mg/mL), it was 2.3 cm^2, after 24 h. Cast_Gnk2-like pure protein treatment reduced the *P. cinnamomi* growth area by more than 100%. The same pattern was found after 48 h of incubation (Figure 5).

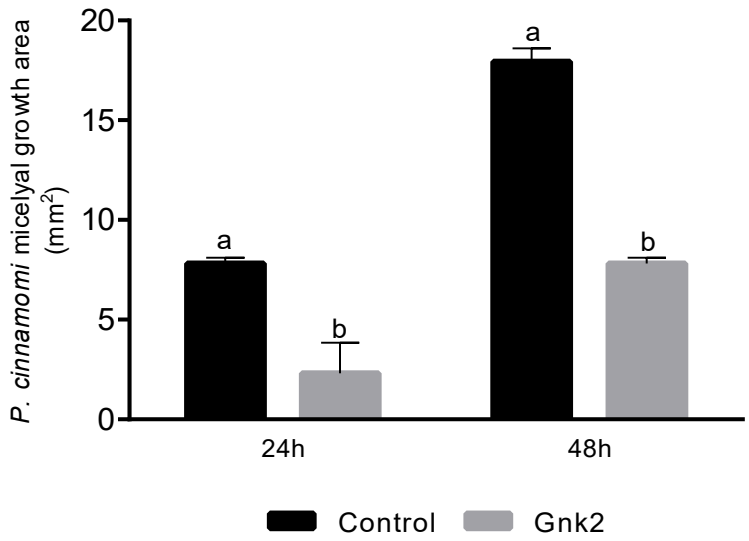


Figure 5. Mycelia area (mm^2) of *P. cinnamomi* growth in PDA under control treatment (only with buffer solution) and Cast_Gnk2-like pure protein treatment (250 µL/plate and 0.5 mg/mL) in Petri plates after 24 and 48 h at 25 °C in darkness. Results are shown as means ± SEM. Significant differences are shown as different letters (*t*-Test, $p < 0.05$).

The dried mycelia of *P. cinnamomi* growing from PDA plugs in potato dextrose liquid medium (after 48 h shaking) under control treatment was visibly higher (Figure 6A) than in Cast_Gnk2-like pure protein treatment, where the mycelia growth was reduced (Figure 6B). The mycelial weight (g) of *P. cinnamomi* under the Control treatment was significantly higher (0.00892 g) than in Cast_Gnk2-like pure protein treatment (0.00187 g) after 48 h in shaking (Figure 7). The Control treatment had an increment in mycelia weight (g) of 377%.

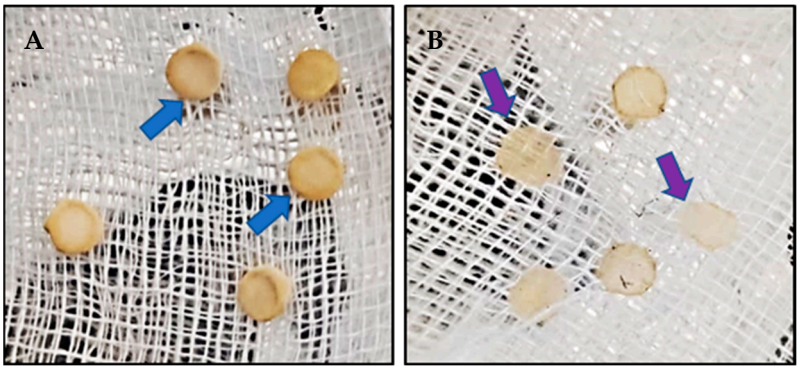


Figure 6. *Phytophthora cinnamomi* mycelia dried (after 24 h at 65 °C) on PDA plugs (5 mm diam.) grown in potato dextrose liquid medium (50 mL) for 48 h in shaking (180 rpm) at 25 °C. Some of *n* plugs are shown as representative of the assay. (**A**): Control treatment (only with buffer solution), and (**B**): Cast_Gnk2-like pure protein treatment (250 µL and 0.5 mg/mL).

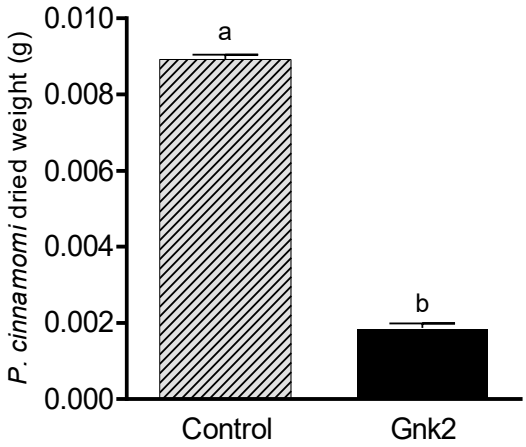


Figure 7. Mycelial dry weight (g) of *P. cinnamomi* growth in potato dextrose liquid medium (50 mL) under control treatment (only with buffer solution) and Cast_Gnk2-like pure protein treatment (250 µL and 0.5 mg/mL) after 48 h in shaking (180 rpm) at 25 °C. Results are shown as means ± SEM. Significant differences are shown as different letters (*t*-test, $p < 0.05$).

4. Discussion

This work is part of the ongoing research program that aims to find solutions to the health problem regarding European chestnuts (*C. sativa*) and the pathogen *P. cinnamomi* [1]. This program involves an integrated approach using genomics, phenomics (precision phenotyping), transcriptomics, association mapping of traits, and histopathology, to reveal the mechanisms of the resistance of Asian species to infection by *P. cinnamomi*, and to transfer the knowledge for the improvement of resistance of European chestnut [7,9,13,18–22]. The program is supported by a breeding program initiated in 2006, based on controlled crosses, from which segregated hybrid populations were obtained with the selection of new genotypes with improved resistance to *P. cinnamomi*, which soon will be disclosed to the market to be used as rootstocks. The research and breeding efforts made in the last decades are having a positive impact since chestnut production in Europe has been increasing since 2015 for the first time in many decades. Nevertheless, more research and techniques are needed to overcome the decline of European chestnut [1].

The secretion of antimicrobial proteins in plants and the reinforcement of the cell wall are part of constitutive defense barriers against pathogens [7,23]. Also, the secretion of compounds toxic to pathogens is an effective chemical defense mechanism in plants. According to Santos et al. (2017) [7], Ginkbilobin-2 (Gnk2) is the most expressed protein, secreted by *Ginkgo biloba* seeds [24], and it was shown to possess antifungal activity. In a

previous study where we used a histological approach to observe the responses exhibited by susceptible and resistant chestnuts under *P. cinnamomi* infection, we found that the early accumulation of phenolic-like compounds in cell walls was observed 0.5 h after inoculation in *C. crenata* root tissues which may prevent the spread of hyphae [9]. In the chestnut species resistant to *P. cinnamomi*, *C. crenata*, the Ginkgobilobin-2-like gene (isolated from transcriptomes of *C. crenata* [13] showed relevant expression constitutively and upon *P. cinnamomi* inoculation when compared with the susceptible *C. sativa* [7]. These previous studies of our group focused on the resistance of chestnut and its response to the Oomycete, indicating that *C. crenata* Ginkgobilobin-2-like gene and the encoded protein (Cast_Gnk2-like) might be important in chestnut resistance. In an in vitro tolerance assay with the pathogen *P. cinnamomi*, Serrazina, et al. (2022) [20], observed that transgenic plants of holm oak (*Quercus ilex*) with the Cast_Gnk2-like were able to survive longer than non-transgenic ones [20]. Similar results were obtained in resistance tests against *F. oxysporum* in transgenic cucumber (*Cucumis sativus*) plants, which showed that the expression of Gnk2-1 conferred antifungal activity against the disease [25]. Currently, to further demonstrate Cast_Gnk2-like action, the group is working with somatic embryos of *C. sativa* lines genetically transformed with the overexpression of the gene. These are being maintained for further assays (studies are still in process).

Other authors have also reported the antimicrobial effects of this protein against many plant and human pathogens [10,25]. However, Cast_Gnk2-like was never tested against *P. cinnamomi* pathogen to improve the defense focused in Chestnut/*P. cinnamomi* pathosystem.

The present work focused on expressing the Cast_Gnk2-like protein and testing its efficacy as a control of *P. cinnamomi*. The first step was to optimize the protein expression and test its effectiveness against the pathogen. The recombinant protein expression was performed following different conditions to optimize its process, and the best expression was obtained using the *E. coli* strain BL21 (DE3) pLysS. These results are in correspondence to the previous work of Miyakawa et al. (2007) [26], where the Gnk2 was overexpressed in *E. coli* BL21(DE3) using pET-26b and pDsbABCD1 vectors [25].

Diseases caused by soil-borne pathogens (such as ink disease) are difficult to eradicate, because of their asymptomatic nature during the initial stages of infection and their complex modes of life and dissemination of the pathogens [27]. The possibility of cure or eradication is very low, and it is, therefore, necessary to develop multidisciplinary strategies to reduce and control them. Farmers depend basically on fungicides to control these diseases. For many years synthetic fungicides were used to control plant production decay. However, public concerns about the harmful effects of chemicals on human health and the environment have caused scientists to search for new alternatives. The incidence of pathogens infections might become more severe in the context of climate change with the possible rise of new strains. For that, it is important to start making more efforts toward these problems [1].

Currently, we are developing studies and tests for the *in-silico* protein structure prediction, in vitro 7-day antagonist assays with *P. cinnamomi*, and metabolomics analysis.

This work re-confirmed that the protein studied has significant potential for biotechnological developments that could reduce the destructive impact of these harmful diseases. Moreover, the Cast_Gnk2-like gene is demonstrated to be a valuable candidate for marker-assisted selection of tolerant *C. sativa* genotypes. Finally, methods and strategies for the production of antimicrobial phytopharmaceuticals considering Cast_Gnk2-like (pure protein) against *P. cinnamomi* are also being considered for further research.

5. Conclusions

This work is part of the ongoing research program that aims to find solutions for European chestnut decline mainly due to *P. cinnamomi*, the causal agent of ink disease. The possibility of eradicating this pathogen is extremely low, and it is necessary to develop multidisciplinary strategies to reduce and control it. Our group is studying new approaches and developing novel techniques for this purpose. As a result of this research, we determined

that Cast_Gnk2-like has a signal peptide that guides the final protein peptide to the apoplast. Moreover, Cast_Gnk2-like (pure protein) significantly reduced the mycelia growth of *P. cinnamomi* in vitro, indicating the potential antagonistic effect on this pathogen. These insights reveal the potential of Cast_Gnk2-like for agricultural uses and biotechnological developments, which are becoming more necessary and critical in managing the threat this pathogen possess to European chestnut under rapidly changing environmental conditions.

Author Contributions: Conceptualization: R.L.C. and M.B.C.; Methodology: F.V.D., S.S. and M.B.C.; Validation: R.L.C., M.B.C., F.V.D. and S.S.; Formal analysis: R.L.C., M.B.C., F.V.D. and S.S.; Investigation: M.B.C., F.V.D. and S.S.; Resources: R.L.C. and R.M.; Data curation: M.B.C., F.V.D. and S.S.; Writing—original draft preparation: M.B.C.; Writing—review and editing: R.L.C., F.V.D., S.S., R.M. and M.B.C.; Visualization: R.L.C., F.V.D., S.S., R.M. and M.B.C.; Supervision: F.V.D. and R.L.C.; Project administration: R.L.C.; Funding acquisition: R.L.C. and R.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Fundação para a Ciência e Tecnologia under the project 414103 FCT-Lx-FEDER-28760, DL57/2016/CP [12345/2018]/CT[2477] to S.S., DL57/2016/CP [12345/2018]/CT[2470] to F.V.D., UIDB/04046/2020, UIDP/04046/2020, UIDB/04046/2021 and UIDP/04046/2021 to BioISI.

Data Availability Statement: Data from this paper are not included in any other site or repository.

Acknowledgments: We acknowledge Helena Machado (Instituto Nacional de Investigação Agrária e Veterinária, I.P) for providing Phytophotora cinnamomi culture for inoculations and Alan Phillips (BioISI—BioSystems and Integrative Sciences Institute) for English review and valuable suggestions.

Conflicts of Interest: The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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