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Biochemistry

Antimicrobial Activity of Greater Celandine (*Chelidonium majus* L.) Plant Seed Lectin

Maia Khurtsidze[*], Nugzar Aleksidze[**], Giorgi Alexidze[§]

* *St. Andrew the First-called Georgian University of the Patriarchate of Georgia, Tbilisi*
***Academy Member, St. Andrew the First-called Georgian University of the Patriarchate of Georgia, Tbilisi*
§ Medical & biological Scientific-Research Center "Alexis", LTD, Tbilisi

ABSTRACT: Content of lectin (CBL-1) in seeds of the Greater Celandine (*Chelidonium mays* L.) was studied. It is demonstrated that in contrast to the total protein, content of CBL-1 is not dependent on the physiological state of seeds (stage of seed maturation and the state of seed dormancy) and it does not change. Content of CBL-1 in the Greater Celandine seeds and the surrounding area within 25 days of germination is of reciprocal character indicating secretion of CBL-1 into surrounding environment since the 20th day of germination. Experiments, conducted in vitro show that CBL-1 causes agglutination and suppression of propagation capacity of the following phytopathogenic microorganisms: *Pectobacterium aroidae, Xanthomonas campestris*, the human pathogenic bacteria *Staphylococcus aureus, Agrobacterium tumefaciens* and the pathogenic fungus *Trichoderma viride*. *© 2015 Bull. Georg. Natl. Acad. Sci.*

Key words: *Chelidonium majus, antimicrobial activity, lectin.*

The primary role of plants in the global ecosystem is widely known. They, together with a small group of bacteria, are the only living organisms, which are capable of synthesis of organic molecules at the expense of the solar energy. Actually, life of all the rest living organisms completely depends on the organic molecules synthesized by plants. Exactly due to this, plants are the most desirable targets for a series of predators and microbes. Scholars argue that plants have their own protection system – phytoimmunity – for defence against microorganisms.

Detection of antimicrobial substances of plant origin, with the aim of their use as antimicrobial agents in the agriculture and medicine, is one of the rapidly developing branches of modern biology, agriculture, ecology and medicine.

Intensive research is being carried out in this direction during the last decade. A series of antimicrobial proteins (AMP were detected, which immediately connect to pathogenic microbial organisms causing inhibition of their growth, propagation and dissemination. Proteins belonging to various classes, possessing antimicrobial properties were isolated from different organs of a number of plants (seeds, bulbs, rhizomes and other tissues). These are: thionins, transport proteins, defencins, chitinases, glucanases, albumins and lectins[1].

Putative role of lectins in plant defence mechanisms is being discussed since the time, when it was discovered that lectins are capable to specifically bind with sugars, locatedon surfaces of microorganisms, causing their agglutination [2].

Aim of the present research was study of antimicrobial activity and possible molecular mechanisms of action of chitin-specific lectin (CBL-1) isolated from the Greater Celandine (*Chelidonium mays*) seeds.

In special experiments we studied:

a) seasonal dynamics of lectin content in the Greater Celandine seeds:

b) ability of the Greater Celandine seeds for secretion of lectins into surrounding area;

c) ability of the Greater Celandine seed lectin for agglutination of microorganisms;

d) antimicrobial activity of the Greater Celandine seed lectin.

Object of Investigation and Methods

Mature seeds of the medicinal plant Greater Celandine - *Chelidonium majus* L. (family Papaveraceae), distributed in Georgia, were used as the object of investigation.

Isolation of CBL-1

Soluble protein fraction was isolated using PBS solution+0.5mMβ-mercaptoethanol (pH 7.4). Ratio of raw material extracted in the solution was 1:40 (g/ml). Homogenization was carried out in a porcelain bowl. Extraction of soluble proteins from the homogenized raw material was done at a room temperature on a magnetic shaker during an hour. The mixture was filtered through the double gauze and the filtrate centrifuged at 16 000 g for 15 minutes. The supernatant was filtered first through the filter (Miracloth, Calbiochem, USA), and then gradually through Whatman GF/c and synpor-0.45μ filters.

For the precipitation of CBL-1 rich proteins, ammonium sulphate was added to the extract of soluble proteins in conditions of constant shaking. After dissolution of the salt stirring was continued for 20 minutes. Protein suspension was left at -10°C overnight and then centrifuged at 20 000 g for 20 minutes at 4°C temperature. Excess ammonium sulphate ions were removed by the dialysis on the G-10 Sephadex gel-filtration column (50x2.7 cm). The sediment was dissolved in the minimum volume of PBS.

Lectin activity was evaluated as minimum concentration of protein (mg/ml) which caused agglutination of Trypsin-treated rabbit erythrocytes. For evaluation of lectin activity **specific activity** (mg/ml) was also determined: $SA=T^{-1}\cdot C^{-1}$, where T^{-1} (Titre) is the degree of protein dilution in the last well of the titration plate, where hemagglutination still occurs ($T=2^n$, n – is number of agglutination wells, C- protein concentration in mg/ml)[3].

Lectin content was judged by the ratio of total protein to the lectin activity (conventional agglutination unit) HU (Hemagglutination Unit).

Specificity of lectins to carbohydrates was studied using hapten-inhibitory method [4]. 0.6 M solutions of oligosaccharides on the basis of PBS were used for analyses. The 22 carbohydrates were used in experiments: D-galactose, methyl-D-galactose, N-acetyl-D-galactosamine, D-mannose, methyl-D-mannose, D-glucose, L-fucose, D-fructose, L-inositol, L-rhamnose, D-xylose, D-arabinose, L-ribose, D-galacturonic acid, D-lactose, D-maltose, saccharose, D-trehalose, D-cellobiose, D-melibiose and D-raffinose as well as oligomers of N-acetyl glucosamine (partially purified hydrolysate of chitin). Carbohydrate solution was titrated with decreasing concentration from 200 mM, on the immunologic titration slide. To all wells of a slide equal concentration of a lectin solution at a titre 1:4 was introduced. Hapten-specificity was judged by the minimum concentration of a carbohydrate (mM), which caused inhibition of hemagglutinating activity of a lectin [4].

Hydrolysis of colloidal chitin (Chitin practical grade powder obtained from Sigma Chemical Co.) was performed in HCl of 7 normality at 40°C for 15 hours.

Table 1. Seasonal dynamics of CBL-1 content in Greater Celandine seed

	Physiological state of seeds				
	Freshly matured seeds	Dormant seeds (seeds were kept in a dry, dark place at room temperature)			
	May, 2013 year	August, 2013 year	November, 2013 year	February, 2014 year	May, 2014 year
C (mg/ml)	7.21	6.01	4.61	1.91	1.33
HA	0.00097	0.00095	0.00075	0.00031	0.00022
LC	7432	7300	6146	6161	6045

HA-hemagglutination activity; LC- lectin content(LC = C/HA)

HCl was removed by evaporation and the hydrolyzate was dissolved in the PBS.

Protein concentration was determined by the method of Lowry [5] using the graph calibrated on the basis of bovine serum albumin(BSA, Sigma).

Antimicrobial activity was tested using the disc-diffusion method [6]. Bacterial and fungal strains, used in experiments, were obtained from the Department of Microbiology, Virology and Biotechnology of the Faculty of Natural Sciences and Medicine of Sokhumi State University.

The following pathogenic bacteria *Pectobacterium aroidae, Xanthomonas campestris, Agrobacterium tumefaciens, Staphylococcus aureus* and also pathogenic fungi – *Trichoderma viride* and *Candida albicans* were used in experiments.

The 18-24 hour-old bacterial and fungal cultures were applied in experiments at 10^5-10^6 cfu/ml (colony forming units calculated per 1 ml of the solution), 50-100 µl of which were homogeneously spread over the surface of 2% agar by the spatula. After drying the bacterial and fungal cultures their surfaces were covered by sterile discs of filter paper (Whatman N3, 6 mm diameter). On the latter the purified protein fraction (protein concentration 66.5 µg/ml) was poured in the amount of 25 µl. In control samples of bacterial and fungal cultures, in one case the discs were saturated by the 1% solution of kanamycin and in another case, the sterile discs were saturated with the PBS solution. Sterile Petri dishes were placed into thermostat, at 28ºC temperature, for 18-24 hours in case of bacteria, and for 48-72 hours in case of fungi. By comparison of the results obtained from the experimental and control Petri dishes the degree of suppression of bacterial and fungal growth was evaluated on the basis of sizes of sterile zones, formed around discs, measured in mm.

Agglutination of Bacteria

Bacteria were washed several times with TBS, at 3000 rpm for 5 minutes. Bacteria were treated overnight at 4ºC with 1.5% (v/v) glutaraldehyde in TBS, then washed 2-3 times and aldehyde groups were blocked with 1M Glycine at 4000rpm for 5 minutes, then at 6000 rpm for 7-8 minutes, then the sediment was added by 2-2 ml of glutaraldehyde + PBS and kept in the fridge for 12 hours; next day it was again washed several times with 1M Glycine and TBS. Bacteria dissolved in TBS were placed on a shaker in order to obtain homogenous bacterial suspension.

Optical density for all three bacteria was equal to 0.1 on the spectrophotometer (КФК-3) at 585 nm, and the lectin concentration was 0.01 mg/ml. Initially, for obtaining an optimal result the lectin+bacteria were taken at different ratios (the control – bacteria+ PBS, PBS mixture was taken with the same ratios – in particular, 1/4, 1/8, 1/10, 1/14, 1/20. Incubation period was different as well - 2, 3, 4, 5, 6, 8 and 12 hours. Smears were stained with fuchsin. Agglutination was checked using the light microscope, at x40 magnification. Optimum conditions, in which complete agglutination of bacteria with lectins took place, turned out to be 1/14 and 1/20 dilution, and

Table 2. The dynamics of CBL-1 content in the Greater celandine seeds and surrounding environment during the 25 day period of germination

Greater celandine seeds and surrounding environment	Protein concentration, C (mg/ml)	Hemagglutination activity, HA (mg/ml)	Lectin content LC
Non germinated	1.33	0.00022	6 045
Germinated (1-15 days)	-	-	-
Germinated (20^{th} day)	0.93	0.00121	768
Germinated (25^{th} day)	1.21	0.004	302
Environment, surrounding the gernminated seeds (20^{th} day)	0.25	0.0013	192
Environment, surrounding the germinated seeds (25^{th} day)	0.75	0.00025	3 000

incubation time – 8-12 hours.

Germination of the Greater Celandine Seeds

The four sets (4 parallels) of hundred seeds of Greater Celandine were taken, weighed (weight of 100 seeds was approximately 2 mg) and left in the water flow for 24 hours to imbibe. Seeds were surface-sterilized in the solution of $KMnO_4$ for 5 min and 80% ethanol (for 10 min). For stimulation seeds were placed into Murasige-Skoog's solution for 24 hours. Seeds were germinated on Petri dishes of sterile cotton wool or filter paper, in the thermostat at 26ºC temperature.

Results and Discussion

According to the data, available in scientific literature the content of lectins in plant organs is variable and often correlates with this or that biological process [7].

In the first series of experiments seasonal dynamics of CBL-1 (HU) content in the Greater Celandine seeds was studied. Content of lectins in seeds was determined during the year, every 3^{rd} month.

As Table 1 shows, the total content of protein in the Greater Celandine seeds decreases 5.4-times starting from June to May, including the latter. The same Table shows, that content of CBL-1 in seeds during the whole period remains practically unchanged. The obtained results indicate that in contrast to the total protein the content of CBL-1 in seeds is not dependent on the physiological state of seeds (stage of seed ripening and state of dormancy) and remains unchanged.

Lectins are known to be mobile proteins and their content in different plant organs correlates with physiological processes proceeding in them. Establishment of such correlation is a step forward, in order to prove the biological role of lectins.

The results obtained by us, in particular, correlation of CBL-1 content with the state of dormancy, undoubtedly points to the biological role lectins may play in seeds being in the dormant state. Number of scientists suppose, that lectins, which accumulate in seeds, play storage (accumulation) role and they are used as a stock of amino acids at the stage of seed germination [7]. According to another suggestion, seed lectins have a protective function, as very often they reveal antimicrobial activity against the phytopathogenic microbes [1].

Proceeding from the above said, the dynamics of CBL-1 content on the next physiological stage was of special interest for us.

In special experiments we studied dynamics of CBL-1 content in Greater Celandine seeds and surrounding environment within the 25 day of germination (Table 2, Fig. 1).

Table 2 shows, that content of CBL-1 in seeds remains actually unchanged on the 5^{th}, 10^{th} and 15^{th} days of germination and equals (LC=6045). From the 20th day of germination a sharp decline of CBL-1 content is marked (LC=768) dropping to the minimum on the 25^{th} day (LC=302).

As seen from the same Table, presence of CBL-1

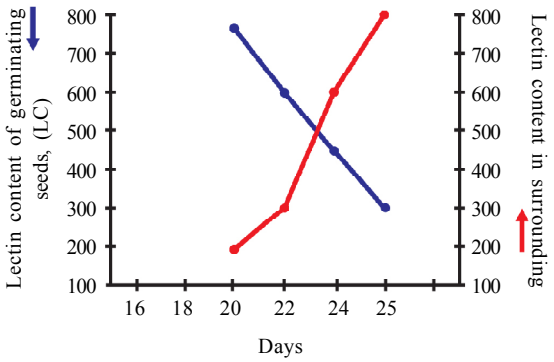


Fig. 1. Dynamics of lectin content in germinating seeds of the Greater Celandine and the surrounding environment during the period of germination

in the environment, surrounding germinating seeds, was not registered on the 5th, 10th and 15th days. CBL-1 content in surrounding area was fixed on the 20th day (LC=192), and its maximum content was registered on the 25th day (LC=3000).

Reciprocal dependence of CBL-1 content in the Greater Celandine seeds and surrounding environment of the stage of germination within 25-day period of germination (Fig. 1) indicates that from the 20th day of germination, secretion of CBL-1 from seeds into environment takes place.

In the next series of experiments we studied specificity to sugars of the Greater Celandine seed lectin CBL-1 and the lectins, isolated from the 20th day of germination from the area, surrounding germinating seeds.

As seen from Table 3, lectins, isolated from seeds and those, registered from the environment, surrounding germinating seeds, reveal identical specificity to carbohydrates, in particular, only to chitin. The obtained results prove identical nature of lectins, registered in seeds and those appearing in the environment, surrounding seeds since the 20th day of germination.

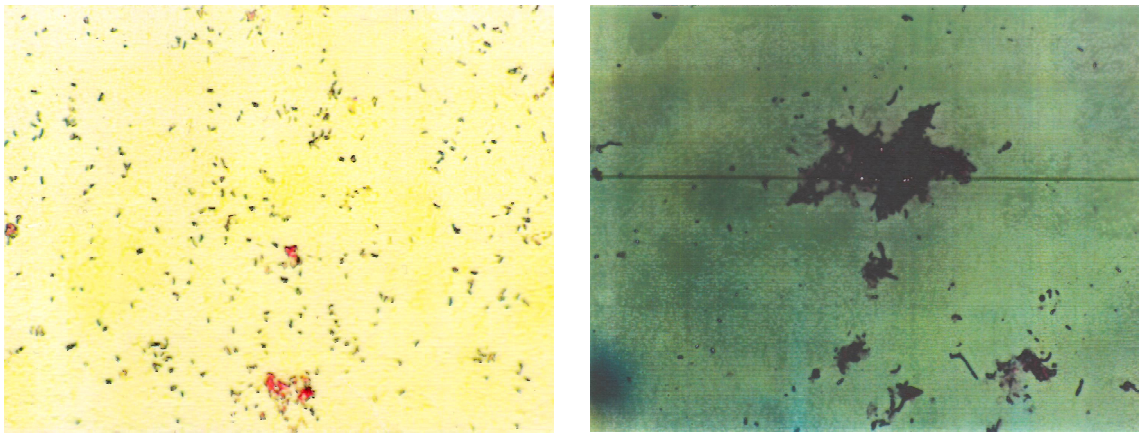
In separate experiments the capacity of microorganisms' agglutination by lectins, fixed in the environment, surrounding germinating seeds was studied. Agglutination was registered visually, using the light microscope.

As seen from Fig. 2, in Control variant, to which CBL-1 was not applied, diffused arrangement of phytopathogenic bacteria takes place, while in the experimental variant, where CBL-1 was used, agglutination or sticking of phytopathogenic bacteria is evident.

In experiments, where test cultures of phytopatho-

Carbohydrates (initial concentration 200 mM)	Inhibition of hemagglutination activity	Minimum inhibiting concentration of carbohydrates (mM)
D-galactose	_	
methyl-D-galactose	_	
N-acetyl-D-galactosamine	_	
D-mannose	_	
methyl-D-mannose	_	
D-glucose	_	
methyl-D-glucose	_	
L-rhamnose	_	
N-acetyl-D-glucosamine L-	_	
L-fucose	_	
D-galacturonic acid	_	
fructose	_	
Oligomeres of N-acetyl-D-glucosamine (hydrolyzate of partially purified chitin)	+	1.562
D-arabinose	_	
L-ribose	_	
D-melibiose	_	
D-lactose	_	
D-maltose	_	
D-trehalose	_	
saccharose	_	

Table 3. Specificity to sugars of the Greater Celandine seed lectin and lectins, isolated from the environment, surrounding germinating seeds,from the 20th day of germination



Control
Bacteria without CBL-1

Expeimental
Agglutinated bacteria in the presence of CBL-1

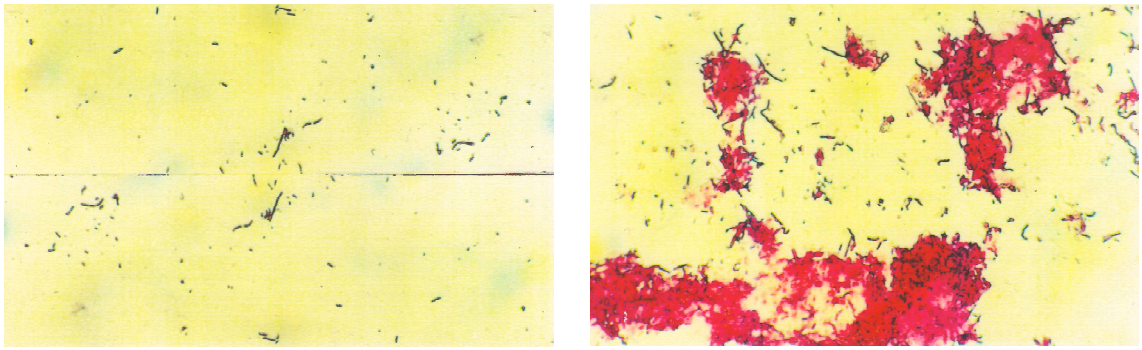
Fig. 2. Visual fixation of agglutination of phytopathogenic bacteria *Agrobacterium tumefaciens* by CBL-1 by light microscope (magnification x 40)

genic bacteria *Pectobacterium aroidae, Xanthomonas campestris, Agrobacterium tumefacien*s, human pathogenic bacteria *Staphylococcus aureus* and pathogenic fungi *Candida albicans, Trichoderma viride*were used, it was demonstrated that CBL-1 caused agglutination of *Pectobacterium aroidae, Xanthomonas campestris, Agrobacterium tumefaciens Staphylococcus aureus* and *Trichoderma viride* and did not reveal agglutination ability towards the pathogenic fungus *Candida albicans*.

In parallel to this, in separate experiments visually, using the light microscope, agglutination of the soil phytopathogenic fungus *Trichoderma viride* by the lectin, fixed in the environment, surrounding germinating seeds was evaluated.

As seen from Fig. 3, in experiments in vitro CBL-1 lectin of the Greater Celandine seed causes severe agglutination of soil pathogenic fungus. Results of investigation showed that CBL-1 causes agglutination of all microorganisms, used in our experiments, except *Candida albicans*.

In special experiments we studied antimicrobial activity of CBL-1 against the test cultures of phytopathogenic bacteria *Pectobacterium aroidae, Xanthomonas campestris, Agrobacterium tumefaciens*, human pathogenic bacteria *Staphylococcus aureus* and pathogenic fungi *Candida albicans* and *Trichoderma viride*. Phytopathogenic microorganisms, used in experi-



Control
Fungal micellia without CBL-1

Experimental
Agglutinated fungal micellia in the presence of CBL-1

Fig. 3. Visual fixation of agglutination of phytopathogenic fungus *Trichoderma viride* by CBL-1 using the light microscope (magnification 40x)

Table 4. Antimicrobial activity of CBL-1, isolated from nongerminated and germinated seeds and from the surrounding environment, at the 20th day of germination

Microorganisms bacteria/fungi	Antimicrobial activity of CBL-1 (inhibition zones in mm)		
	CBL-1, isolated from nongerminated seeds	CBL-1, isolated from germinated seeds	CBL-1, isolated from the environment, surrounding germinating seeds
Bacteria *Pectobacterium aroidae*	14	12	10
Bacteria *Staphylococcus aureus*	18	17	15
Bacteria *Xanthomonas campestris*	10	10	8
Bacteria *Agrobacterium tumefaciens*	12	10	9
Fungus *Trichoderma viride*	6	7	5
Fungus *Candida albicans*	–	–	–

ments are known to be characterized by a wide spectrum of harmful effects against such agricultural crops, as carrot, tomato, salad, spicy herbs, onions, green pepper, pumpkin, etc. Diseases, caused by the mentioned microorganisms are accounted worldwide as the most destructive (harmful) diseases [8-12]. Human pathogenic bacteria *Staphylococcus aureus* is a gram-positive bacteria, which is the most widespread staphylococcus, often appearing in the human respiratory tract and skin. It is established that 25-40% of the population are the long-term carriers of *S. aureus*. This bacteria may cause such life-threatening diseases as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, sepsis and others [13]. Nowadays treatment of heavy diseases, caused by *S. aureus* is one of the most urgent problems of the contemporary medicine because of their resistance to antibiotics [14].

CBL-1, isolated from the 20th day of germination from the area, surrounding nongerminated and germinated seeds, was used in experiments for the determination of antimicrobial activity.

Data, presented in Table 4 show that CBL-1, isolated from nongerminated and germinated seeds as well as that isolated from the environment, surrounding these seeds from the 20th day of germination, revealed strong antimicrobial activity to all bacterial test-cultures, used in the experiments. CBL-1 reveals antifungal activity towards the fungus *Trichoderma viride*, though it did not reveal such activity against the fungus *Candida albicans*. The obtained results indicate that CBL-1 (66.5 μg/ml), isolated from three listed sources, reveals practically identical antimicrobial activity.

Thus, since the 20th day of germination the seeds of the Greater Celandine start secretion of CBL-1 into surrounding area and cause agglutination and suppression of growth, propagation and spreading of bacteria *Pectobacterium aroidae, Xanthomonas campestris, Agrobacterium tumefaciens,* human pathogenic bacteria *Staphylococcus aureus* and phytopathogenic fungus *Trichoderma viride*.

Results of the study give an idea on molecular mechanisms of antimicrobial action and protective functions of CBL-1 lectin, isolated from the Greater Celandine seeds. The Greater Celandine seeds lectin CBL-1 belongs to the class of plant antimicrobial proteins (AMP). The obtained results allow tosupposethat in natural conditions in the wild protective endogenous role of the Greater Celandine seed lectin is protection of germinating seeds from the harmful action of phytopathogenic microorganisms, occurring in the surrounding soil.

Antimicrobial properties of CBL-1 against phytopathogens can be used in practice for protec-

tion of a wide range of agricultural crops and their products. It is known that phytopathogenic organisms, susceptible to the antinmicrobial activity of CBL-1: *Pectobacterium aroidae, Xanthomonas campestris, Agrobacterium tumefaciens* and *Trichoderma viride* cause serious economic damage to the agriculture and ecosystem at a global scale.

Data of the research demonstrated possibility of treatment with CBL-1of infectious diseases, caused by the human pathogenic bacteria *Staphylococcus aureus*. This makes CBL-1 a prospective tool for application in pharmacological industry and clinical medicine.

ბიოქიმია

მცენარე ქრისტესისხლას (*Chelidonium majus* L.) თესლის ლექტინის ანტიმიკრობული აქტივობა

მ. ხურციძე[*], ნ. ალექსიძე[**], გ. ალექსიძე[§]

* *საქართველოს საპატრიარქოს წმიდა ანდრია პირველწოდებულის სახელობის ქართული უნივერსიტეტი, თბილისი*

***აკადემიის წევრი, საქართველოს საპატრიარქოს წმიდა ანდრია პირველწოდებულის სახელობის ქართული უნივერსიტეტი, თბილისი*

§ *მედიკო-ბიოლოგიური სამეცნიერო-კვლევითი ცენტრი „ალექსის“, შპს, თბილისი*

შეწავლილია ქრისტესისხლას თესლებში ლექტინის (CBL-1)-ის შემცველობა. ნაჩვენებია, რომ სუმარული ცილებისაგან განსხვავებით, CBL-1-ის შემცველობა არ არის დამოკიდებული თესლების ფიზიოლოგიურ მდგომარეობაზე (თესლების მომწიფების სტადია და მოსვენების მდგომარეობა) და იგი უცვლელია. CBL-1-ის შემცველობა ქრისტესისხლას თესლებსა და მათ გარემომცველ არეში გაღივების 25-დღიან პერიოდში რეციპროკული ხასიათისაა და მიუთითებს, რომ გაღივების მე-20 დღიდან ადგილი აქვს CBL-1-ის სეკრეციას თესლებიდან გარემომცველ არეში. ნაჩვენებია, რომ CBL-1 *in vitro* ექსპერიმენტებში იწვევს ფიტოპათოგენური მიკროორგანიზმების: *Pectobacterium aroidae, Xanthomonas campestris*, ადამიანის პათოგენური ბაქტერია *Staphylococcus aureus, Agrobacterium tumefaciens* და *Trichoderma viride*-ს აგლუტანაციას და მათი ზრდისა და გამრავლების უნარის დათრგუნვას.

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