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BRIEF COMMUNICATION

A latex lectin from *Euphorbia trigona* is a potent inhibitor of fungal growth

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Abstract

In this study we identified and characterized a major latex lectin – designated as EtLLH – with antimicrobial activity from the succulent African milk tree *Euphorbia trigona*. The lectin is highly concentrated in the latex of *E. trigona* and appears to be composed of at least two subunits with a molecular mass of 32 kDa. EtLLH shares significant similarities to known plant lectins – ricin from *Ricinus communis* and agglutinin from *Viscum album coloratum* – which specifically bind D-galactose and N-acetyl-D-glucosamine, the major building blocks of many fungal cell walls. Antimicrobial activity assays revealed an impact of EtLLH on three phytopathogenic filamentous ascomycetes. The germination of the conidiospores and the hyphal growth of *Aspergillus niger* and *Fusarium graminearum* were severely inhibited by EtLLH already at concentrations below 0.1 mg cm^{-3}, while the effect on germination of the melanized conidiospores of *Botrytis cinerea* was less significant.

Additional key words: African milk tree, antimicrobial activity, *Aspergillus niger, Botrytis cinerea*, *Fusarium graminearum*, phytopathogenic fungi, sugar-binding protein.

Latex is commonly known as a milky to transparent sap and is produced in more than 12 500 plants (Kekwick 2007). The biological function of latex in diverse plant families remains elusive, however, several authors suggested a role in defence against microbial and herbivore attacks (Lewinsohn 1991, Wahler *et al.* 2009). Some plant species including *e.g. Hevea brasiliensis* (Van Parijs 1991) and *Pedilanthus tithymaloides* (Seshagirirao 1995), are known to produce lectins in their latex. Plant lectins are a complex and heterogenous group of proteins or glycoproteins with diverse molecular structures and biochemical properties that bind to sugar moieties of complex glycoconjugates (Souza *et al.* 2005, for review see Chrispeels and Raikhelb 1991, Peumans and Van Damme 1995, De Lucca *et al.* 2005). Lectins seem to play an important role in the defence mechanisms of plants against the attack of microorganisms. Interactions of lectins with fungi were first demonstrated by Mirelmann *et al.* (1975). They observed that wheat germ agglutination (WGA), a lectin specific for chitin oligosaccharides, inhibits hyphal growth and spore germination by binding to the hyphal tips and septa of a chitin containing fungus. Other known lectins affecting fungal growth are for example *Urticola dioica* agglutinin (UDA), hevein from the latex of the *Hevea brasiliensis* (Van Parijs *et al.* 1991), *Solanum tuberosum* agglutinin (STA) and gastrodianin antifungal proteins (GAFPs; for review see De Lucca *et al.* 2005). Different lectins could also be isolated, purified and characterized from the latex of several members of the *Euphorbiaceae* family including *Hura crepitans* and *Euphorbia characias* (Barbieri *et al.* 1983), *E. calcina*, *E. dalberi* L. (Nsimba-Lubaki *et al.* 1986), *E. neriifolia* (Guerrero and Guzman 2004) and *Synadenium carinatum* (Souza *et al.* 2005). Here, we present the molecular characterisation of a latex lectin with antifungal activity from *E. trigona*. The antifungal activity of the lectin was determined by the inhibition of the conidiospore germination rate and the

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Abbreviations: LC-MS - liquid chromatography - mass spectrometry; SDS - sodium dodecylsulphate.
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hyphal growth of the filamentous fungi *Aspergillus niger*, *Botrytis cinerea* and *Fusarium graminearum* causing severe pre- and postharvest diseases in plants.

Euphorbia trigona Miller was cultivated in the greenhouse of the botanic garden at the Westphalian Wilhelms-University of Münster. The filamentous fungi *Aspergillus niger* (DSMZ 2466), *Botrytis cinerea* B05.10 and *Fusarium graminearum* strain P1 were grown on a solid CM medium (Leslie and Summerell 2006) at 28 °C (*F. graminearum, A. niger*) or at room temperature (*B. cinerea*) for one week.

Latex from *E. trigona* was harvested after wounding the stem with a rasor blade. The crude latex fraction was mixed in a ratio of 1:3 with saline-phosphate buffer (1× PBS: 2 mM sodium phosphate buffer, pH 7.4, 15 mM NaCl) and subsequently centrifuged at 10 000 *g* for 5 min at 4 °C in order to separate the aqueous phase from the main latex particles. The protein concentration in the pellet and supernatant was determined after filter dialysis with Mr 3000 size exclusion columns (*Sartorius*, Göttingen, Germany) following established protocols (Bradford 1976). For SDS-PAGE analysis the protein samples were mixed with either reducing (dithiotreitol, β-mercaptoethanol) or non-reducing protein loading buffer, heated to 100 °C for 10 min and separated on a 10 % gel in running buffer at 30 mA for 2 h. For determination of the molecular mass, proteins were visualized by staining with 0.25 % (m/v) Coomassie brilliant blue and compared to the precision blue protein standard (*Bio-Rad Laboratories*, München, Germany).

For mass spectrometry analysis, the protein band was excised out of the SDS-Gel and digested in-gel according to Mortz *et al.* (1994). Therefore, the gel plug was destained by incubating in an aqueous solution of 15 mM potassium hexacyanoferrate and 50 mM sodium thiosulfate. Digestion was performed overnight at 37 °C in 0.05 cm^3 50 mM NH_4HCO_3 containing 1 µg trypsin (sequencing grade, modified, *Promega*). The resulting peptide digests were analysed using LC-MS/MS according to Naumann *et al.* (2005). The *OMSSA* MS/MS search engine (Geer *et al.* 2004) and the *MS-BLAST* (Shevchenko *et al.* 2001) were used for sequence filtering and database search. Computer-assisted alignment of protein sequences was performed with *ClustalW2* (Larkin *et al.* 2007).

For the antimicrobial activity assay, conidiospores from on cultivated *F. graminearum, A. niger* and *B. cinerea* were obtained by diluting conidiospores containing agar pieces in 1 cm^3 1× PBS buffer and filtration through fibreglass. Concentrations of conidiospores were determined using a hematocytometer and adjusted to 7.5×10^5 conidiospores cm^{-3}. 96-well microtitre plates were filled with a spore suspension (0.01 cm^3 per well) followed by the addition of different volumes of latex protein (final concentration of 0.0, 0.01, 0.05, 0.1 and 0.5 mg cm^{-3}). Finally, each well was supplemented with 0.09 cm^3 of malt extract peptone medium (malt extract 30 g dm^{-3}, peptone 3 g dm^{-3}, pH 5.6). The plates were then incubated for 24 h at 28 °C (*F. graminearum, A. niger*) and room temperature (*B. cinerea*), respectively. The germination and the hyphal growth were assessed under a *Leica* binocular microscope (magnitude 11×; Wetzlar, Germany). The hyphal growth was evaluated with a measuring eyepiece. All experiments were repeated 6 times if not stated otherwise. Germination rate was determined from at least 250 conidiospores, whereas the hyphal growth was measured from at least 32 independent germinated conidiospores.

For the characterisation of the protein composition, latex from the stem of *E. trigona* was collected and subjected to SDS-PAGE analysis. Only one dominant protein was detected in the soluble fraction with a molecular mass of either ~32 kDa under reducing (Fig. 1*A*) or ~60 - 70 kDa (Fig. 1*B*) under non-reducing conditions. It should be noted that little amounts of the same protein were also identified in the pellet fraction (data not shown). Most plant lectins are composed of several homogenous or heterogenous subunits with molecular masses between 26 - 400 kDa (Souza *et al.* 2005). Depending on the analysed species, two or more subunits of Mr 28 - 32 kDa have been identified for *Euphorbiaceae* lectins (Nsimba-Lubaki *et al.* 1986, Seshagirirao and Prasad 1995, Souza *et al.* 2005). According to the differences in the molecular mass obtained under reducing and non-reducing conditions a contribution of at least two subunits in the formation of the dominant latex protein of *E. trigona* seems to be likely. Subsequently, the 32 kDa protein band was excised from the Coomassie stained gel and analysed by mass spectrometry after tryptic digestion. The MS fragmentation pattern was used to perform *de novo* sequencing and the obtained putative peptides were analysed with the *OMSSA* software. Peptides with reliable score and e-value were compared with public databases using the *MS-BLAST* algorithm. About 50 putative peptides showed significant identities (high scoring peptides, HSP, score > 75) to known lectins from different plants, whereas up to four different peptides

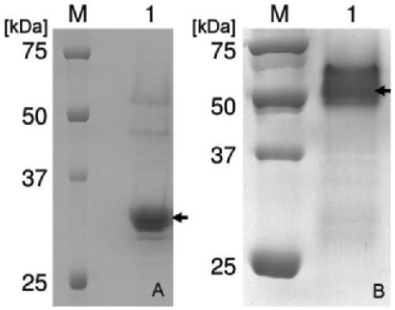

Fig. 1. Protein content of *E. trigona* latex. The crude latex extract was centrifuged for 5 min (10 000 *g*) and 0.025 cm^3 of the supernatant was mixed with reducing (*A*) and non-reducing (*B*) protein loading buffer and subjected to SDS PAGE. *Lane* M - molecular mass marker.

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RC   ADVCMDPEPIVRIVGRNGLCVDVRDGRFHNGNAIQLWPCKSNTDANQLWTLKRDNTIRSN 60
VCA  DVTCTASEPTVRIVGRDGLCVDVRDGKFYNGNPIQLWPW----DPNQLWTIRRDGTIGSN 56
       .*  .** ******:*********:*:***.*****     * *****::**.** **
ET                                                QLWTLASDGTIQSD
                                                  ****:  *.** *:

RC   GKCLTTYGYSPGVYVMIYDCNTAATDATRWQIWDNGTIINPRSSLVLAATSGNSGTTLTV 120
VCA  GRCLTTYGYTAGVYVVIFDCNTAVREATLWQIWGNGTIINPRSNLVLGAASGSSGTTLTV 116
     *:*******:.****:*:*****. :** ****.*********.***.*:**.*******
ET   GK
     *:

RC   QTNIYAVSQGWLPTNNTQPFVTTIVGLYGLCLQANSGQVWIEDCSSEKAEQQWALYADGS 180
VCA  QTQVYFLGQGWLAGNDTAPREVTIYGFGNLCMEANGASVSVETCGGSKENQKWALYGNGS 176
     **::* :.****. *:* *  .** *: .**::**...* :* *...* :*:****.:**
                                                  QPQQWYFYPDGT
                                                    *:* :* :*:

RC   IRPQQNRDNCLTSDSNIRETVVKILSCGPASSGQRWMFKNDGTILNLYSGLVLDVRASDP 240
VCA  IRPKQNQDQCLTSQGDSVSTVFNIVSCSAGSSGQRWEFTNEGTILNLNNGLVMDVAQSNP 236
     ***:**:*:****:.:  .**.:*:**...****** *.*:****** .***:**  *:*
ET   IRPK                                         NVLVMDVAHSKP
     ***:                                         . **:**  *.*

RC   SLKQIILYPLHGDPNQIWLPLF 262
VCA  SLRRIIIYPATGKPNQMWLPVP 258
     **::**:**  *.***:***:
ET   SLK IIVYPFTGNPNQK
      **: **:** :*.***
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Fig. 2. Alignment of *de novo* sequencing derived EtLLH peptides to the B-chain of lectins from *Ricinus communis* (RcR, NCBI accession No. P02879) and *Vicum album coloratum* (VcA, NCBI accession No. P06750). Protein and peptide sequences were aligned with the *ClustalW2* program. Identical residues (*), conserved substitutions (:) and semiconserved substitutions (.) are indicated.

Table 1. Effect of the addition of latex fractions with different EtLLH concentrations on the germination rate [%] and hyphal growth [mm] of the conidiospores of *A. niger*, *B. cinerea* and *F. graminearum.* After 24 h incubation at respective temperature, germination rate was examined and the hyphal growth was evaluated with a measuring eyepiece. Each data point represents the mean and standard deviation of at least 250 analysed conidiospores for the germination rate and 32 for the hyphal growth, respectively.

EtLLH protein [mg cm^{-3}]	*A. niger* germination rate	hyphal growth	*B. cinerea* germination rate	hyphal growth	*F. graminearum* germination rate	hyphal growth
0	100	5.2 ± 0.6	100	4.2 ± 0.3	100	6.1 ± 0.5
0.01	91 ± 2	5.0 ± 0.4	98 ± 2	3.7 ± 0.4	96 ± 3	5.6 ± 0.5
0.05	72 ± 4	2.4 ± 0.2	95 ± 1	3.0 ± 0.3	82 ± 3	3.6 ± 0.3
0.10	52 ± 3	0.7 ± 0.2	87 ± 3	2.4 ± 0.2	65 ± 1	1.9 ± 0.3
0.50	0 ± 4	0.0 ± 0.0	72 ± 4	1.8 ± 0.2	19 ± 4	0.5 ± 0.1

(HSP sum scores > 300) could be aligned to one lectin (Fig. 2). Thus, the identified latex protein was designated as *E. trigona* latex lectin homologue (EtLLH).

Highest identities within a range between 56 - 75 % for four peptides were obtained for the B-chain of ricin from *Ricinus communis* (RcR) and of agglutinin from *Vicum album coloratum* (VcA). Both lectins specifically bind to galactose and N-acetyl-D-glucosamine, which are major building blocks of many fungal cell walls. Active ricin is a dimeric glycoprotein (65 kDa), which is composed of a toxic A-chain (31 kDa) that is linked to a sugar-binding B-chain (34 kDa) by a disulphide bound. The B-chain is responsible for the binding to glycoproteins at cell surfaces (Olsnes and Kozlov 2001). For EtLLH, we suggested a similar protein composition and a role in plant defence.

Thus, we set out to determine if EtLLH exhibit antimicrobial activity by adding filter dialysed soluble latex fractions with different protein concentrations of EtLLH to conidiospores from *A. niger, F. graminearum* and *B. cinerea* in liquid medium (Table 1). Compared to the corresponding water controls EtLLH caused an

inhibition of the conidiospore germination rate for all three fungal species. Similar results have been already observed for other plant lectins showing that these proteins may possibly serve as fungistatic agents in the plant kingdom, especially during spore germination and early fungal growth (Ciopraga *et al.* 1999). Other studies described that the germination is only retarded by adding a plant lectin (Barkai-Golan *et al.* 1978).

In the case of *A. niger* and *F. graminearum* the inhibition of germination and growth in the presence of EtLLH decreased in a concentration dependent manner. In contrast, *B. cinerea* germination was only slightly affected even at higher EtLLH concentrations. EtLLH appeared to have the strongest effect on *A. niger* since germination of conidiospores was fully inhibited at EtLLH concentration of 0.5 mg cm^{-3}. At the same EtLHH concentration the other two analyzed fungi still show germination rates of ~20 % (*F. graminearum*) and ~75 % (*B. cinerea*). A similar inhibitory effect could also be observed in the analysis of hyphal growth of all investigated fungi. A 50 % reduction of hyphal growth were obtained at EtLLH concentrations of ~0.5 mg cm^{-3} (*B. cinerea*), ~0.075 mg cm^{-3} (*F. graminearum*) and ~0.05 mg cm^{-3} (*A. niger*) again indicating the weaker potential of EtLLH to efficiently inhibit *B. cinerea* growth. It has been described in literature that different lectins show high specificity for different compounds of the fungal cell wall. Examples are the wheat germ agglutinin (WGA), a sugar-binding protein specific for N-acetyl-D-glucosamine, and peanut agglutinin (PNA), which is more specific for D-galactose. EtLLH shared the highest similarities to lectins, which specifically bind D-galactose and N-acetyl-D-glucosamine. During fungal development the chemistry of the cell wall of spores changes remarkably. Thus, cell wall components such as chitin or galactose residues of young spore surfaces can be replaced or overlayed by other compounds like melanin, which prevent their interaction with lectins (Barkai-Golan *et al.* 1978). Recently, melanin was found to be the major component of the extracelluar matrix of *B. cinerea* (Doss *et al.* 2003). This might explain the different inhibition rates of the conidiospore germination between *B. cinerea* and the other two investigated fungi. While the effect on germination of *B. cinerea* was less significant the inhibition of hyphal growth was much more severe. This obviously demonstrates on the one hand the general anti-fungal activity of EtLLH towards growing hyphae and on the other hand the change of chemical composition of the fungal cell wall and extracellular matrix.

Taken together, EtLLH seems to be a potent inhibitor of germination of non-melanized conidiospores and hyphal growth of filamentous ascomycetes. Future studies will include the elucidation of the protein structure and binding specificities as well as its potential application in organic pre- and postharvest plant protection.

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