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## Cork Warts on Leaves of *Gnetum* L. (Gnetaceae) and its Phylloplane Fungi

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### ABSTRACT

The cork warts on leaves of plants appear to be a response to mechanical injuries or pathogen penetrations. Many of *Gnetum* species regularly form cork warts on leaf surfaces and stems. We have searched anatomy, morphology and development of cork warts and also estimated evaluation of probable influence to its origin by phylloplane fungi. Leaves of two species of Gnetaceae family *G. gnemon* and *G. montanum* have been investigated for anatomical and morphological studies of cork warts and for mycological research. Herbarial specimens of 13 *Gnetum* species have been searched. We have successfully extracted 15 species of phylloplane ascomycetes that appear to influence negatively on continuity of leaf epidermis and being able to existing as parasites as well. The most frequent species were *Cladosporium cladosporioides* which became a dominant indicating 100% of frequency index and representatives of *Fusarium* and *Phoma* genera. The frequency index of latter numbered 36 and 20% correspondingly. Five species of *Penicillium* ascomycetes were also determined as frequent. Cork warts have been found in 13 species of *Gnetum* that grow in natural environment. Cells in local areas of epidermis and subepidermal layers proliferate periclinally during cork wart development in leaves of *G. gnemon* and *G. montanum*. As a result, the layer of high compactly packaged cells emerges. Tannins inside cell compartments and suberinization of cell walls were indicated for cork warts. It emphasizes a defensive function of the structures. Cork warts appear to originate like "Patches" on the surfaces of leaves of evergreen gnetum plants.

**Key words:** Epidermis, blade surface, fungal colonization, cell divisions, adaptation

### INTRODUCTION

The presence of cork warts in leaf blades of angiosperms has been noticed in the end of 19th century (Bachmann, 1880; Keller, 1890; Matteucci, 1897). The term "Cork wart" was introduced by Solereder (1908) as structure that resembles lenticels and consists of a hemispherical group of suberized cells.

Now a days there is no common opinion about causes of origin, development and anatomical structure of cork warts (Metcalf and Chalk, 1950; Morretes and Venturelli, 1985). It is thought that cork warts derive due to proliferation of the stomatal subsidiary cells (Borzi, 1886; Farooqui, 1982),

epidermal cells, basal cells of fell off trichomes (Keller, 1890; Farooqui, 1982) or cells of mesophyll (Borzi, 1886; Keller, 1890; Joffily and Vieira, 2010; Evans and Bromberg, 2010). The division pattern resulting in cork wart formation is attended by suberinization of the walls and partial death of the cells (Farooqui, 1982). The majority of the authors, however, have not been using the term "Periderm" concerning to cork warts. This could be explained by the absence of its typical elements that are phellogen, phellem and phelloderm (Haberlandt, 1928; Stace, 1966; Joffily and Vieira, 2010; Guimaraes *et al.*, 2011).

Some authors connect the origin of cork warts with pathogen invasion into the leaves (Ross, 1896; Dickison,

2000). Its cells appear to isolate the pathogen from vital tissue protecting it. According to another point of view the cork wart emergence could be a response to mechanical injuries (Stace, 1966; Farooqui, 1982). In some cases cork warts originate in plants that grow in specific localities. As an example, the warts were described in the trees of mangrove from the following Rhizophoraceae, Sonneratiaceae, Plumbaginaceae and Acanthaceae families (Stace, 1966; Evans *et al.*, 2009; Evans and Bromberg, 2010). In some species like *Kandelia candel* and *K. obovata* cork warts arise from epidermal cells (Sheue *et al.*, 2003). Other species such as *Rhizophora mangle* and *Rh. racemosa* have the ones originated inside the leaf aerenchyma. Its gradual growth leads to destruction of an epidermis (Evans and Bromberg, 2010). Cork warts are thought to play an important role in internal tissues aeration of the mangrove plants due to Knudsen flow (Evans *et al.*, 2009; Evans and Bromberg, 2010). Some authors had been named the cork warts as lenticels (Haberlandt, 1928; Matteucci, 1897).

Among the seed plants that regularly form cork warts are the majority of species of genus *Gnetum*. These structures have been discovered in leaf blades, petioles and stems of *G. gnemon*, *G. montanum* and *G. ula* (Nautiyal *et al.*, 1976). In the course of leaf structure investigation we have found the cork warts in some other species of *Gnetum* that grow in natural environment and in introduction (Pautov and Pagoda, 2015). It gives an excellent opportunity to explore the warts under greenhouse conditions.

This article is devoted to research of anatomical structure, origin and development of the cork warts of *Gnetum* species and evaluation of probable influence to its origin by phylloplane fungi.

Phylloplane is the surface of the leaf blades of higher plants. It is a specific habitat for a diversity of microorganisms: bacteria, filamentous fungi, yeasts and algae (Prabakaran *et al.*, 2011; Saha *et al.*, 2013; Borgohain *et al.*, 2014). The main sources of its nutrients are excretions of plants and substances percipating from the atmosphere. Some of the phylloplane organisms are parasites of the plants (Inacio *et al.*, 2002). Micromycetes of phylloplane play the most significant role in this community (Langvad, 1980). *Alternaria alternata*, *Cladosporium cladosporioides*, *Gliocladium viridae*, *Mucor racemosus*, *Penicillium chrysogenum* are the species that have the widest distribution in the leaf surfaces of plants. These species are inhabitants of wide spectrum of plant species around the world (Saha *et al.*, 2013).

## MATERIALS AND METHODS

**Leaf sampling and processing:** Leaves of two species of Gnetaceae family (tree *Gnetum gnemon* L. and liana *G. montanum* Markgr) were investigated. Plant material was collected in greenhouse No. 20 of botanical garden of

Komarov Botanical Institute Russian Academy of Science (RAS), Saint Petersburg, September 2014. Collected leaves were fixed in 70° ethanol.

Herbarial specimens were collected from herbarium of Department of Botany, Saint Petersburg state University (LECB) and herbarium of Komarov Botanical Institute RAS (LE), Saint Petersburg. There were examined 13 species of genus *Gnetum*: *G. africanum* Welw., *G. funiculare* Brogn., *G. gnemon* L., *G. indicum* (Lour.) Merr., *G. latifolium* Bl., *G. laxifrutescens* Elm., *G. leyboldii* Tul., *G. loerzingii* Markgr., *G. montanum* Markgr., *G. paniculatum* Spruce ex Benth., *G. philippinense* Warb., *G. scandens* Roxb. and *G. ula* Brogn (Table 1). Herbarial material was collected in January, 2012 and March, 2014. We researched an epidermis of leaf blades and petioles, an anatomy of cork warts and a diversity of phylloplane fungi. The duration of the fungi research has been planned for period of 2014-2016.

### Research in anatomy and morphology of leaves

**Softening of herbarial specimens:** Small fragments of leaves were cut out from herbarial specimens and put into weighing bottles with distilled water for 2-6 h. We used the mixture of glycerin, distilled water and ethanol (70°, 96°) in proportion of 1:1:1 for softening of dried leaf fragments. The weighing bottles with plant material in mixture were put into thermostat under 60-70°C for 24 h. Then the material was washed by distilled water and used for next manipulations.

**Maceration method:** The fragments of epidermis were received with maceration method. The specimens of leaves were put into mixture of full-strength nitric acid (HNO<sub>3</sub>) and potassium chlorate (KClO<sub>3</sub>) on 1-1, 5 h, then washed by distilled water. Next, the ones were put in mixture of potassium chloride (KOH) and distilled water on 30-60 min, then washed by distilled water.

Separation of upper and lower epidermis was provided by using preparation needle under stereomicroscope Leica EZ4. We used safranin for staining cell walls of epidermis.

**Preparation of microscopic sections:** For making transverse microscopic sections of leaf blades and petioles their fragments were embedded with paraffin (Barykina *et al.*, 2000).

Firstly, the material was dehydrated in series of ethanol with increasing concentrations (30°, 50°, 70°, 80°, 90°, 96°, 100°) for 30 min in each one.

Secondly, the specimens were impregnated by β-lemonen (bioclear, the hydrophobic substance) throughout using ethanol (100°)-β-lemonen mixture of following ratio: 3:1, 1:1, 1:3, then two times in pure β-lemonen. Impregnation lasted 60 min in each substance.

Thirdly, the fragments were filled up with paraffin-β-lemonen mixture (24 h in thermostat under 55°C) and paraffin only (7 days in thermostat under 55°C).

Table 1: Herbarial specimens from herbarial collections of Saint-Petersburg State University (LECB) and Komarov Botanical Institute RAS (LE)

Species	Location	Leg.	Determ.	Year
<i>G. africanum</i>	Cameroon	G. Zenker	G. Zenker	1896
<i>G. funiculare</i>		Finllet	Finllet	1887
<i>G. gnemon</i>	Ceylon, Perodeniya	V.V. Markovich	V.V. Markovich	1927
<i>G. gnemon</i>	New Guinea, Lae	B.G. Briggs	B.G. Briggs	1970
<i>G. indicum</i>	Ting Hong-Shang mount, China	A.L. Takhtajan	A.L. Takhtajan	1958
<i>G. indicum</i>	Hainan, Sun-To District, near Tai Chung village	W.T. Tsang		1933
<i>G. indicum</i>	Hainan, Lam Ko-Chang Mai	W.T. Tsang	E.D. Merrill	1928
<i>G. indicum</i>	Lantao Island	Y.W. Taam	Y.W. Taam	1941
<i>G. indicum</i>	Shatin, Kowloon Chuck-Pok-Hang	Y.W. Taam	E.D.M.	1940
<i>G. indicum</i>	Hong Kong Island	Y.W. Taam	Y.W. Taam	1940
<i>G. indicum</i>	Sumatra island, East coast	E.D. Merrill	H.S. Yates	
<i>G. indicum</i>	Hainan Ch'ang-Kiang District	S.K. Lao	E.D. Merrill	1933
<i>G. indicum</i>	Philippines, Bokol Philippines, Sibuyan island	M. Ramos	M. Ramos	1923
<i>G. laxifrutescens</i>	Prov. Capiz, E. Kudryavtseva, G.	E. Kudryavtseva,	A.D.E. Elmer	1910
<i>G. latifolium</i>	Vietnam, Quang Ninh, Vietnam, prov. Tuyei	Ogureeva USSR-Vietnamian	G. Ogureeva	1990
	Quang	Expedition	N.T. Hiej	1961
<i>G. latifolium</i>	Vietnam, Yia Lai Kontum S. Vietnam, prov. Kontum, Binh, A. Budantzev	LX-VN L. Averyanov, N.Q.	N.T. Hiej	1985
<i>G. latifolium</i>	Ngoc Linh Mount System Vietnam, Tuyeu Quang	G. Yakovlev		1995
<i>G. latifolium</i>	Hyouq lap Philippines, Sibuyan island		N.T. Hiej	1961
<i>G. latifolium</i>	Capiz sumatra island, east coast, asahan, Mashih forest	A. D. E. Elmer	A. D. E. Elmer	1910
<i>G. loerzingii</i>	Reserve Sumatra island, Eastcoast	B.A. Krukoff	B.A. Krukoff	1932
<i>G. loerzingii</i>	Asahan	V.A. Krukoff		1932
	China, prov. Yunnan	Expeditio Biologica		
<i>G. montanum</i>	Manshi Mount	Sino-Rossica L. Averyanov		1955
		N.Q. Binh, P.K.		
<i>G. montanum</i>	Vietnam, Ninh Thuan, Vietnam, Ninh Thuan	Loe L. Averyanov, N.Q. Binh, P.K.	N.T. Hiej	1997
<i>G. montanum</i>	Dalat sity	Loe G.T. Prance	N.T. Hiej	1997
<i>G. nodifolium</i>	Brazil, Rio Moa Brazil, San Carlos, Rio	P.J.M. Maas	F. Markgraf	1973
<i>G. paniculatum</i>	Negro Philippines, Luson island, prov. Beguet, locality	G. Spiriu		1923
<i>G. philippinense</i>	Bagulo	A.D.E. Etmer	A.D.E. Etmer	1907
<i>G. scandens</i>	Hong Kong, Junio	H.F. Hance	H.F. Hance	1866
<i>G. scandens</i>	Hong Kong, Lantao island	Y.V. Taam	E.D.M.	1941
<i>G. scandens</i>	China, Yunnan	A. Henry	A. Henry	
<i>G. scandens</i>	Hong Kong, Junio	H.F. Hance	H.F. Hance	
<i>G. scandens</i>	Hab. Khasia, Regio Hop.	J.D.H. C. Saldanha	J.D.H. C. Saldanha	1856
	India, Hassan District	T.P.	T.P.	
<i>G. ula</i>	Mysore	Ramamoorthy	Ramamoorthy	1971

The microscopic sections were obtained by using of microtome SAKURA Accu-Cut SRM 200. The reverse manipulations with sections were made after cutting (washing up with bioclear, rehydration in ethanol of decreasing concentrations, washing up in distilled water).

The sections were stained by combined staining agent alcian blue and safranin. All specimens were embedded in glycerin-gelatine medium in microscope slide and preserved under cover glass.

**Specific staining:** The staining of cork warts on suberin was produced by potassium chloride (KOH) which stained them in bright yellow color (Barykina *et al.*, 2000). The fragments of blade and petiole epidermis with cork warts and its transverse sections were heated in 30% solution of potassium chloride.

Tannins were found by staining the cork warts with Kartis safranin which colored them in dark red (Prozina, 1960). The specimens were put into weighing bottles with safranin solution and kept in thermostat under 60°C for 30-90 min. Then they were cooled down under indoor temperature and

washed by acetic acid (CH<sub>3</sub>COOH) for 3 min. The fragments were stained next in alcian blue for 5 min and washed firstly with acetic acid on 3 min, secondly with distilled water and embedded in glycerin-gelatine medium.

**Making a photographs:** Photographing of material under the cover glass were produced with microscopes Leica DM500 and Leica DM1000, camera Leica EC3. Photographs were converted in Leica Application Suite software (Las EZ), Leica Microsystems Framework.

**Scanning electron microscopy:** Epidermis of leaves and structure of cork warts were investigated by method of Scanning Electron Microscopy (SEM). The fragments of leaf blade and petiole were dehydrated in series of ethanol of with increasing concentrations (20°, 50°, 70°, 80°, 90°, 96°, 100°). Next, the ones were filled into mixtures of acetone and ethanol (100°), acetone and isoamyl acetate and isoamyl acetate only. Dehydrated specimens were dried under critical point of fluid carbon dioxide (CO<sub>2</sub>). Dried objects were stucked on stages

and sprayed with ions of gold. We used scanning electron microscope JSM-6390LA for examination of prepared specimens.

### Phylloplane search

**Fungi sampling, plating and identification:** The leaves of *G. gnemon* were taken from middle part of the crone, the ones of *G. montanum* were collected from high, middle and low parts of liana stem. The sampling consisted of 10 leaves for *G. gnemon* and 9 leaves for *G. montanum*. We searched both abaxial and adaxial leaf surfaces. Mycological samples were collected by three different ways.

Firstly, the fragments of colonies and fungal structures (conidia and mycelia) were transferred from leaf blade to petri dishes with agar by sterile preparation needle (point isolation). We used this way for plating phylloplane fungi from abaxial leaf surface only. Samples were collected along main vein, secondary veins and from fragments of blade surface with indicators of fungal existence (developed dark-colored mycelia and fruiting hyphae).

Secondly, the surfaces of leaf blades were treated by sterile swabs on the fragments with dark bloom indicating fungal existence. Leaf blades were previously screened under stereomicroscope.

Thirdly, fungal fragments were transferred on the nutrient solution by method of impression replicas. Two leaf blades of *G. gnemon* and 3 ones of *G. montanum* were treated this way. Impression replicas were taken from whole abaxial blade surfaces of *G. gnemon* and from regions of main vein and secondary veins of *G. montanum* leaves.

Plating were taking place in petri dishes on Czapek Dox Agar. The identification was provided after germination and forming of colonies (Fig. 1a and b). The fungal species were identified on the basis of cultural characteristics and morphology of fruiting bodies and spores by using standard texts and keys. The species was then identified by using the identification manual (Bilay and Koval, 1988; Ellis, 1971, 1976; De Hoog and Guarro, 1995; Pidoplichko, 1977, 1978; Satton *et al.*, 2001).

## RESULTS

**Anatomy of cork warts:** We have examined 13 species of genus *Gnetum* and found cork warts on surfaces of its leaves: *G. africanum*, *G. funiculare*, *G. gnemon*, *G. indicum*, *G. latifolium*, *G. laxifrutescens*, *G. leyboldii*, *G. loerzingii*, *G. montanum*, *G. paniculatum*, *G. philippinense*, *G. scandens* and *G. ula* (Table 1). All of them have been collected from natural habitat. Identical structures have been noticed in *G. gnemon* and *G. montanum* introducing in botanical garden of Komarov Botanical Institute RAS (St. Petersburg). Thus, the cork warts have been found in representatives of genus *Gnetum* under natural conditions and in plants growing in botanical garden. It has given us an opportunity to research the structure and development of cork warts of introduced plants as model objects. We also have estimated possible causes of their formation on leaves of *Gnetum*.

The cork warts of *G. montanum* are frequent mostly on lower surfaces of the leaves. This structures are distributed along large veins (main vein and secondary veins predominantly) (Fig. 2a) and in petiole as well. Rarely the cork warts develop between large veins on both surfaces of leaf blades and in epidermis under minor veins. In paradermal section the cork warts have rounded or ellipsoid shape (Fig. 2b and c). The basal square is from 0,012-0,248 mm<sup>2</sup>.

In leaves of *G. gnemon* cork warts are mainly associated with epidermis of petiole. They were also indicated in both surfaces of the leaf blade. The cork warts are distributed solitary or in groups in petioles and in blades. Their shape is the same as in *G. montanum*. The basal area is from 0,18-0,29 mm<sup>2</sup>. The warts of petiole are bigger.

Development of cork warts occurs in young and mature leaves. Initially, on local areas of leaf blades periclinal divisions of epidermal cells and cells of inner tissue occur (Fig. 3a and 4a). The regular rows and layers of cells are forming in the course of this divisions (Fig. 3b, c and 4b). Thus, the cork wart is exerted to be superducted under epidermal surface by pressing of mechanical exertion. The cells of external layers stop to divide first (Fig. 3c). The ending

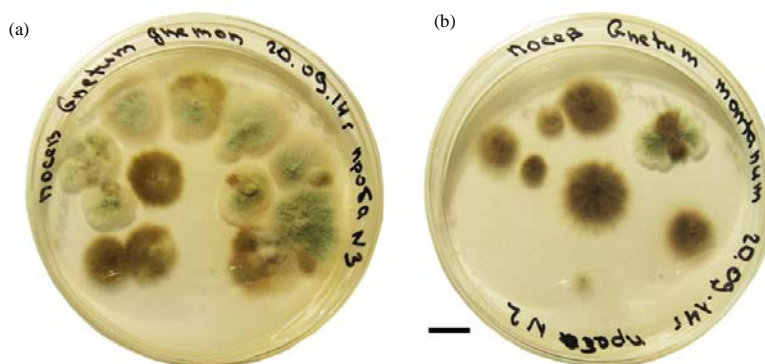


Fig. 1(a-b): Growth of colonies of micromycetes plated from the surfaces of leaf blades of (a) *Gnetum gnemon* and (b) *G. montanum* in petri dishes on Czapek Dox Agar. Dark-colored colonies of *Cladosporium* fungi are the dominants Scale: 1 cm



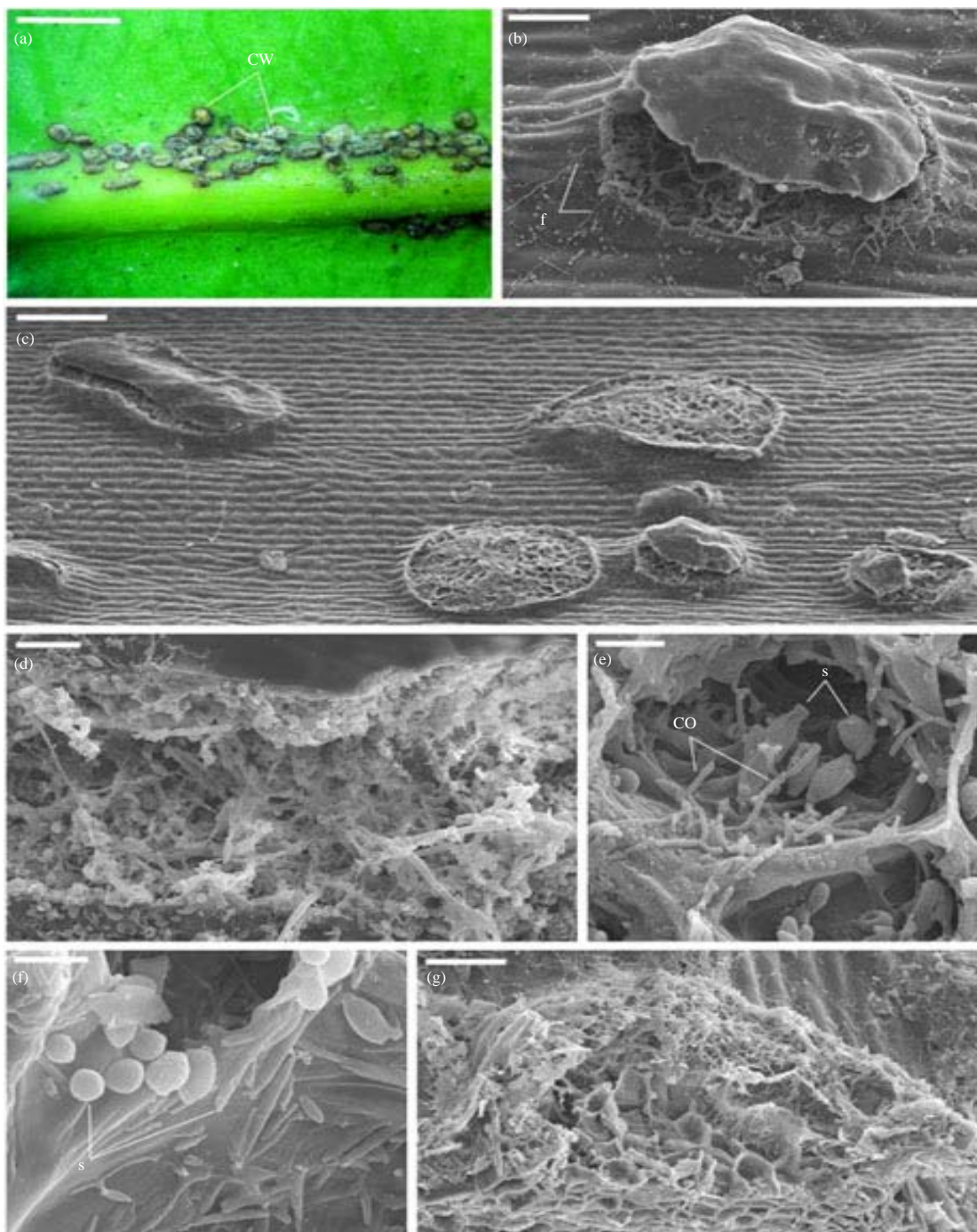


Fig. 2(a-g): Results of SEM research of cork warts in leaves of *Gnetum gnemon* and *G. montanum* (a) Distribution of cork warts along main vein on abaxial surface of leaf blade in *G. montanum* (stereomicroscope view), (b) Cork wart on the abaxial surface of leaf blade in *G. montanum* with cavity filled up by developing hyphae and spores of micromycetes, (c) Surface of leaf blade in *G. montanum* with cork warts, lack of cuticular cap is in some of them, (d) Pattern of micromycetes in the cavity of cork wart in leaf blade of *G. gnemon*, where *Cladosporium* species are dominants forming vegetative and generative structures, (e-f) Mycelia and spores of *Cladosporium* that grow in compartment of dead half-disrupted cells of cork wart in leaf blade of *G. montanum* and (g) The disturbing of cuticle in cork wart of *G. gnemon* leaf blade in zone of intensive growth of micromycetes, CO: Conidia, CW: Cork warts, f: Fungal mycelia, s: Spores, a: 0, 25 cm, b, c: 50  $\mu$ m, d: 200  $\mu$ m, e: f-5  $\mu$ m, g: 20  $\mu$ m

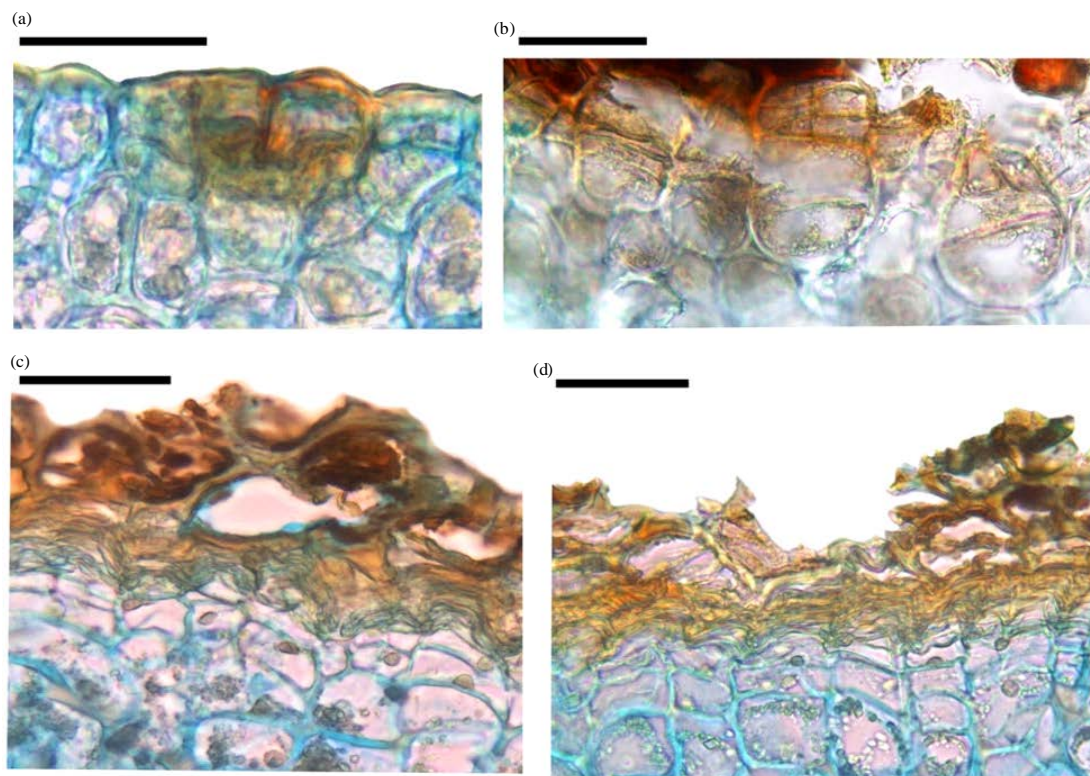


Fig. 3(a-d): Transverse sections of cork warts development in leaf blade of *Gnetum montanum* (light microscopy), (a) Origin of cork wart, initiation of epidermal cells proliferation, (b) Periclinal divisions of cells in inner tissue of cork wart and (c-d) Anatomy of cork wart: proliferating tissue (below), obliterating and dead cells (above), a, c, d: 50  $\mu$ m, b: 20  $\mu$ m

of the divisions is combined with accumulation of tannins in its compartments (Fig. 5a and b). The suberinization of cell walls also occurs (Fig. 5c). Then, these cells die. The cells of inner layers continue to proliferate during some while (Fig. 4b-d). As a result of this division the cell mass enlarges and presses on external layers leading to increasing of cork wart superduction under the blade surface (Fig. 4c, d). Herewith, the cuticle covering of the growing cork wart tears off (Fig. 2b and c). A gap is formed at the base of superducing part of cork wart near the blade surface. External layers of dead cells are deformed and decayed (Fig. 4b and c). The gaps are also formed at the top part of cork wart opening the inner cavity with its compartments.

The cork warts of petiole and blade surface develop in similar way. They appear as a result of epidermal and subepidermal cell divisions (Fig. 6b and c). The cells resulting from this process accumulate tannins and its cell walls are suberinising.

In some cases the epidermal cells not only divide during the development of cork wart but have intensive growth. Its stretching is perpendicular to blade surface (Fig. 6d). The warts with this cell property are highly subducted under the leaf surface. As time goes by some cells are being disrupted.

**Phylloplane fungi:** There have been extracted and determined 13 species of micromycetes from phylloplane of *G. gnemon*: 6 species were on abaxial surface of the leaves, 10- on abaxial one (Table 2). There have been indicated 10 species of fungi derived from blade surface of *G. montanum*: 4 species were on upper epidermis, 8- on lower one (Table 2). All determined species belong to ascomycetes. Figure 7 and Table 2 show the index of fungal occurrence. Besides, there were *Mycelia sterilia* in petri dishes of searching samples. It grows like light-colored or dark-colored fungi isolates without fruiting hyphae or conidia.

*Cladosporium cladosporioides* became a dominant phylloplane ascomycete (Fig. 1). It have 100% index of frequency (Fig. 7). It was represented on both surfaces of the leaf blades in all of the samples where it usually became a dominant or formed a monoculture. Genus *Penicillium* showed the highest level of biodiversity comparable with other ones (Fig. 7). It consisted of 5 species, the most frequent was *Penicillium brevicompactum* (48%) and *P. decumbens* (24%). In few samples *Phoma* sp. were a dominant. Its index of frequency is 36%. *Fusarium* sp. prevailed in all samples collected by method of impression replicas (20%). The biodiversities of fungi in abaxial and adaxial leaf surfaces are similar (Table 2).



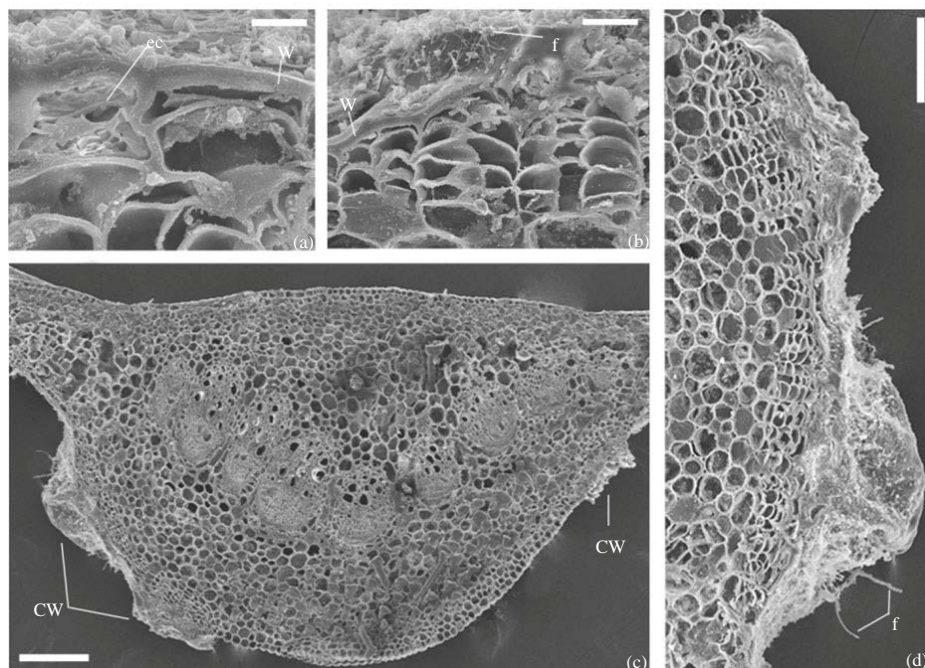


Fig. 4(a-d): SEM results of cork wart anatomy, transverse sections, (a-b) Structure of cork wart tissue with periclinal rows of cells and thick outer cell wall, (c) Middle vein of leaf blade with three cork warts on its abaxial side and (d) Two cork warts with cuticular caps and growing mycelia of phyloplane fungi on it, cw: cork warts, ec: epidermal cell, f: fungal mycelia, w: outer cell wall, a: 20  $\mu\text{m}$ , b: 50  $\mu\text{m}$ , c: 100  $\mu\text{m}$ , d: 200  $\mu\text{m}$

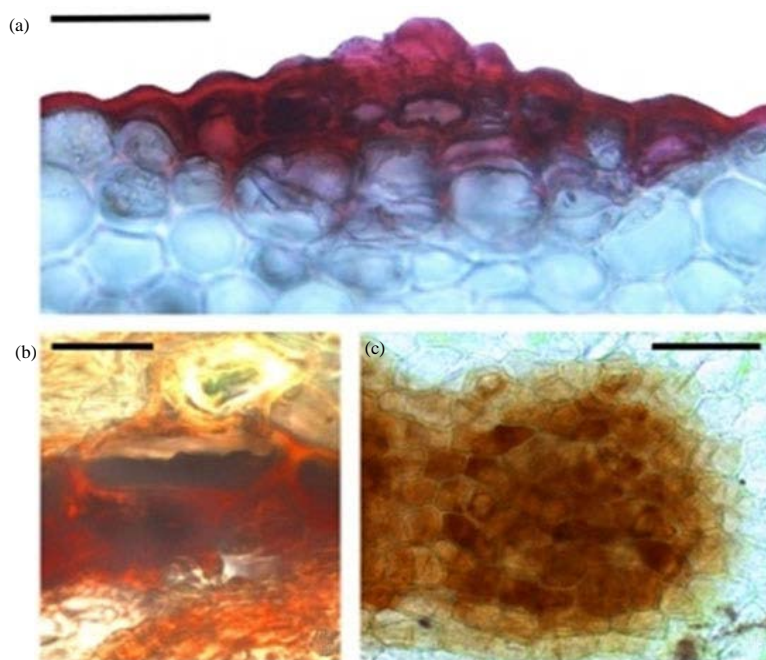


Fig. 5(a-c): Staining for suberin and tannins revealing in tissues of cork warts of (a) *Gnetum gnemon* and (b) *G. montanum* (light microscopy), (a-b) Transverse section of the main vein of leaf with cork wart. Tissue is stained by Kartis safranin that reveals tannins inside cells and in cell walls (red color) and (c) Paradermal section of abaxial surface of leaf blade. Suberin in cell walls is revealed by acetic acid (brown color), a: 50  $\mu\text{m}$ , b: 20  $\mu\text{m}$ , c: 100  $\mu\text{m}$



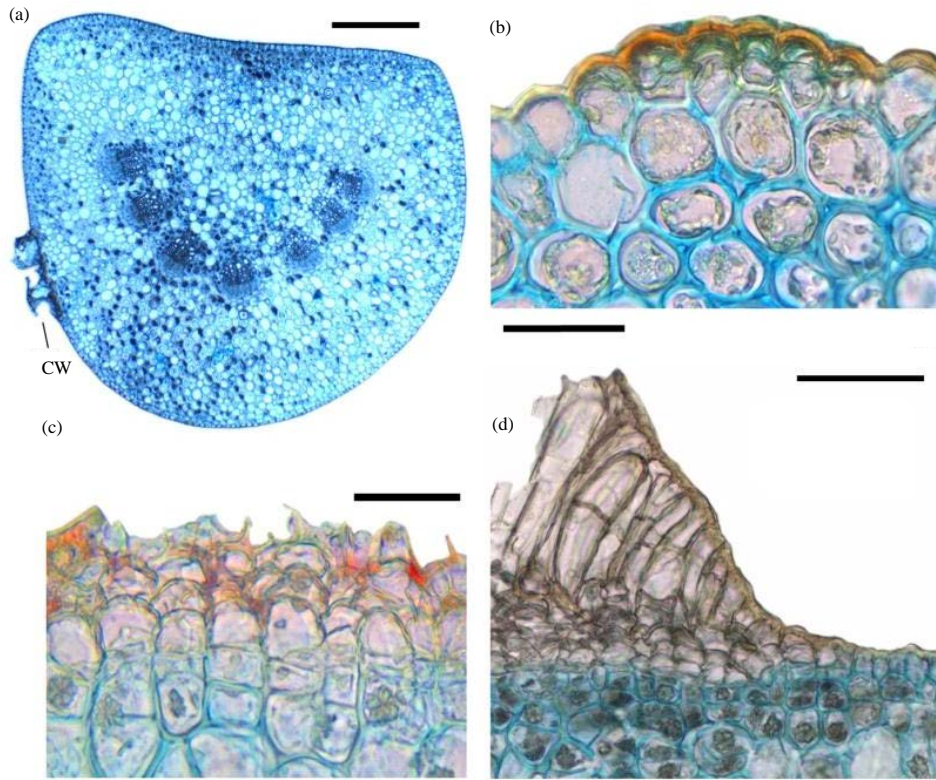


Fig. 6(a-d): Development of cork wart in petiole of (a) *Gnetum gnemon* and (a, c and d) *G. montanum* (light microscopy), (a) Transverse section of petiole with two cork warts on lateral side, (b) Transverse section of originating cork wart; the divisions of epidermal cells are clearly seen, (c) fragment of cork wart with dying, dead and proliferating cells and (d) Mature cork wart with large elongated epidermal cells. This type of cork warts forms only in petiole of *Gnetum* species, CW: cork warts, a: 500  $\mu\text{m}$ , b, c: 50  $\mu\text{m}$ , d: 100  $\mu\text{m}$

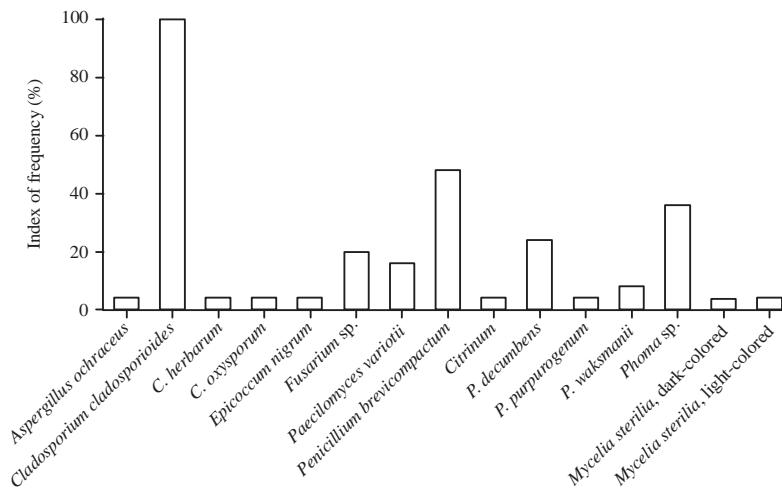


Fig. 7: Index of fungal occurrence in phylloplane of *Gnetum* species, *Cladosporium cladosporioides* is a dominant species (100%). The *Penicillium* genus is the most diversified. It counts 5 species with two of them having high indexes of frequency 48% for *P. brevicompactum* and 24% for *P. decumbens*. *Phoma* sp. and *Fusarium* sp. have indexes of frequency on levels 36 and 20%, respectively

Table 2: Species of micromycetes, revealed due to methods of collecting and subsequent plating in Petri dishes from surfaces of *G. gnemon* and *G. montanum* leaf blades

	Species of fungi	<i>G. gnemon</i>		<i>G. montanum</i>	
		Adaxial surface	Abaxial surface	Adaxial surface	Abaxial surface
1	<i>Aspergillus ochraceus</i> Wilh.	+	-	-	-
2	<i>Cladosporium cladosporioides</i> (Fresen.) G.A. de Vries	+	+	+	+
3	<i>C. herbarum</i> (Pers.) Link	+	-	-	-
4	<i>C. oxysporum</i> Berk. and M.A. Curtis	-	+	-	-
5	<i>Epicoccum nigrum</i> Link	-	-	+	-
6	<i>Fusarium</i> sp.	-	+	-	+
7	<i>Paecilomyces variotii</i> Bainier	-	+	-	+
8	<i>Penicillium brevicompactum</i> Dierckx	+	+	+	+
9	<i>P. citrinum</i> Thom	-	+	-	-
10	<i>P. decumbens</i> Thom	+	+	-	+
11	<i>P. purpurogenum</i> Stoll	-	-	+	-
12	<i>P. waksmanii</i> K.M. Zaleski	-	+	-	+
13	<i>Phoma</i> sp.	+	+	-	+
14	<i>mycelia sterile</i> , light-colored	-	-	-	+
15	<i>mycelia sterile</i> , dark-colored	-	+	-	-
	Total	6	10	4	8

Signs “+” and “-” are marked respectively presence and absence of mentioned phylloplane fungi in samples plated in petri dishes

Comparison of sampling methods used for collecting fungi from leaf surfaces shows that impression replicas is less successful method in revealing of ascomycetes on leaf surface. Direct point isolation of cork wart fragments and mycelia, conidia or hyphae with sterile preparation needle on agar in petri dishes were more prosperous method.

Mycelia, conidia and conidiophores of ascomycetes are shown well on SEM photos (Fig. 2b and 4b). The fungal structures grow on leaf surface directly and in cavities of cork warts, where fungi form a significant biomass (Fig. 2d and g). It is observed that fungal growth is observed straight in cork wart compartments (Fig. 2e and f). The hyphae of micromycetes form dense plexuses inside it and appear to cause a destruction of cuticular layer of cork wart cap, which covers the cavity with common influence of mechanical tension of proliferating cells beneath the base of cork wart (Fig. 4b).

Dominance of representatives of *Cladosporium* genus is significantly depicted under cuticular layer of cork warts (Fig. 2e and f). It forms vegetative and reproductive structures. Mycelia and spores are detected in great number inside the compartments of tumble downed cell walls of dead cells (Fig. 2d). There is growth of single hyphae and separated spores of micromycetes on the surface near the cork warts (Fig. 2b). It is typical for phylloplane of tropical vascular plants. Though density of mycelia increases in the direction of cork warts showing its maximal consistence inside it. Data of SEM research shows in total that there are highly favorable conditions for growth and reproduction of fungi inside cork warts. Obviously in these structures they are protected from external influences and found some nutrient sources.

## DISCUSSION

Many species of fungi have the ability to colonize the leaf surface of angiosperms. Some phylloplane fungi can penetrate

into its epidermis (Kuthubutheen, 1984). Some species of fungi colonized both surfaces of leaf blade. Other ones grow only on abaxial side of the leaf that could be explained by thin cuticular layer here and easier absorption of nutrients from epidermis and mesophyll (Lee and Hyde, 2002).

We have successfully extracted 15 species of phylloplane ascomycetes from *Gnetum gnemon* and *G. montanum* belonging to following genera: *Aspergillus*, *Cladosporium*, *Epicoccum*, *Fusarium*, *Paecilomyces*, *Penicillium* and *Phoma*. The majority of determined ones are associated with abaxial surface of the leaves. Several species are of specific interest. First of all, it is *Cladosporium cladosporioides* as phylloplane dominant of *Gnetum*. It is known as active destructor of various natural substrates and exists as a pathogen that causes plant diseases named cladosporiosis (Kuthubutheen, 1984; Briceno and Latorre, 2008; Saha *et al.*, 2013). It affects plants from different families that have significant agricultural value (tomatoes, vines, wheat, etc.) and ornamental plant (Kuthubutheen, 1984; Lee and Hyde, 2002; Briceno and Latorre, 2008). Other representatives belonging to genera *Fusarium* and *Phoma* are much frequent. The ones can be parasitic for plants at same degree as *C. cladosporioides* (Briceno and Latorre, 2008). Besides there is a significant diversity of *Penicillium* ascomycetes on the surfaces of *G. gnemon* and *G. montanum* leaves. This species can change pH of the surface by excreting organic acids, especially oxalic acid (Magro *et al.*, 1984; Prusky *et al.*, 2004). The assimilation of acids by fungi decreases the steadiness of epidermis against pathogen penetrating (Hadas *et al.*, 2007) and is considered to be the one of the pathogenic factors (Dickison, 2000).

Thus, there are micromycetes in phylloplane of *G. gnemon* and *G. montanum* that appear to influence negatively on continuity of leaf epidermis and being able to parasitism as well.

The representatives of genus *Gnetum* are evergreen plants that grow in tropical rainforests (Cadiz and Florido, 2001;

Tomlinson and Fisher, 2005). The epiphyllic organisms particularly filamentous fungi are active colonizers of phylloplane of plants under tropical conditions (Richards, 1952). The glabrate surface partially prevents the colonization. Another important characteristic of tropical plant leaves is thick cuticle. It defends cytoplasm of epidermal cells and inner tissues of blade and petiole against pathogenic organisms of phylloplane in definite degree. In addition the long-living leaves of evergreen plants are defended in another ways. We suppose the forming of cork warts is one of that ways. We have found this structures in 13 species of *Gnetum* (Table 1) (Pautov and Pagoda, 2015). Its development appears to have been generated by damages of epidermis that had been probably caused by several organisms living in leaf surfaces. As it was noticed above, the organisms like that are existing in phylloplane of young and mature leaves of *Gnetum* species. Thus, we agree with presumptions of the researchers who suppose that cork warts develop as a response to minor damages of leaf surfaces (Dickison, 2000; Joffily and Vieira, 2010; Guimaraes *et al.*, 2011). It is important to note that the cork warts have been described for numerous of angiosperms. But gnetums belong to one of the gymnosperm branches. We did not find any information of cork warts existing in other gymnosperms.

The development of cork warts begins when cells in local regions of epidermis and subepidermal layers of blade or petiole begin to proliferate periclinally. As a result, the layer of high compactly packaged cells emerges. It isolates minor damages and defends vital tissues of leaves against pathogens. The evidence of accumulation of tannins in cells and suberization of cell walls are for benefit of defensive function of cork warts. We emphasize that close-packed cell layers, its suberized cell walls and accumulation of tannins are typical markers of wound cork (Dickison, 2000). Cork warts are like "Patches" on the surfaces of long-lived leaves of gnetums (Fig. 2a-c).

Our results indicate that it is appropriate to use methods of plant anatomy and morphology on the one hand and mycological tools on the other for understanding of composite questions according the interactions of phylloplane fungi and the host plant.

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