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FOLIAR TRAITS INVOLVED IN RESISTANCE TO MELAMPSORA LARICI-POPULINA

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Abstract: *Melampsora larici-populina* Kleban, the causal agent of poplar leaf rust is one of the fungal diseases with the greatest impact on the genus: *Populus*, causing great losses in plantations worldwide. The infestation occurs when the fungus manages to establish itself on the underside of the leaf and penetrate inside it. The morphological differences of the genus Populus, in terms of number of stomata, epithelial cells and size of its leaves, could have an influence on the resistance of this genus to poplar rust. The objective of this study was to evaluate the relationship between the morphological characteristics of Populus (stomata density, epithelial cells and leaf area) and the incidence at *Melampsora larici-populina*. We worked with a total of 50 hybrids from five poplar crosses, stomas, epithelial cells, leaf area, number of leaves were counted for each of them, and the stomatal index and disease incidence were calculated. The results indicate that the evaluated taxa present significant differences in stomatal density and number of epithelial cells per cm2 (p = 0.001). The cross: *Populus trichocarpa* x *P. deltoides* showed the highest number of stomata (NE) per cm2 (22,650 NE/ cm^2) and the highest number of epithelial cells (EC) per cm^2 (107.5 * 104 EC/cm^2). The stomatal index did not show differences in the crosses evaluated. the crossing (*P. trichocarpa* x *P. deltoides*) x *P. deltoides* presented the largest leaf area (203.7 cm2). Regarding the incidence of rust on the crosses evaluated, it was observed that (*P. trichocarpa* x *P. deltoides*) x *P. deltoides* y *P. deltoides*, did not present the disease during the evaluated period, being: *P. deltoides* x *P. nigra,* the taxon that presented the highest incidence with 40 % infection. A very low correlation was observed between the incidence of rust and stomatal density (r^2= 0.13) and the incidence of rust and epithelial cells (r2 = 0.14). On the other hand, a negative correlation was found between incidence and leaf area (r2= -0.19). In summary, the number of stomata, epithelial cells, stomatal index and leaf area differ significantly in the different crosses evaluated. However, no evidence was found that the number of these morphological structures could influence the incidence of poplars when attacked by *Melampsora* spp. For the crosses studied, the anatomical characteristics evaluated are not good parameters in the selection of poplars resistant to rust attack.

Keywords: Populus- poplars- stomata- poplar rust.

INTRODUCTION

The genus: *Populus* presents a high development potential worldwide, for the production of wood, phytoremedial or dendroenergetic crops (Baettig et al., 2010). However, poplars are susceptible to attack by various insects, fungi, bacteria, viruses, and even higher plants. Currently in Chile, one of the most harmful diseases is poplar rust, which is caused by the fungus: *Melampsora larici-populina* Kleb *(*Albornoz *et al.*, 2018). This fungus can cause significant economic losses, especially in low-spaced plantations, where favorable microclimates are created for its development (Pinon and Frey, 2005). It has been quantified that the damage caused by rust can decrease between 30 and 60 % of the growth of poplars (May-de Mio et al, 2006; Pinon et al., 2006).

Melampsora spp., attacks poplar leaves, mostly on the abaxial surface. When the uredospores reach the surface of the leaves susceptible to the fungus, a series of phenomena occurs that lead to the penetration and installation of the mycelium inside the plant (Terhune et al., 1991; Read et al., 1992). Rust penetration into host cells can occur directly or indirectly. According to Ragazzi et al., (2005), the direct penetration is through the epithelial cells, while the

indirect way is through the stomata. Direct penetration is associated with the germ tubes of basidiospores in the monokaryotic state and indirect penetration is associated with the germ tubes of aeciospores and uredospores in the dikaryotic state (Mendgen et al., 1996). However, for Tian et al. (2002), the penetration of the germ tube of *M. larici- populina* inside the poplar leaf is through the stomata. Thus, rust penetration seems to be linked to 1) tactile stimulation, related to the relief of the leaf surface, 2) the presence of stoma guardian cells, and 3) chemical signals that induce the formation of the appressorium to initiate the process of growth. plant infection (Hand and Mendgen, 1997). After the stoma has been invaded, the germ tube develops substomatal vesicles and forms round bodies usually with one or two infection hyphae (Tian et al., 2002; Spiers and Hopcroft, 1988). The hyphae invade the host cells, establishing a high relationship between the host and the parasite (Mendgen and Hahn, 2002).

Poplar leaf rust caused by genus: *Melampsora*, it affects not only native poplars in their respective environments, but also naturally generated hybrids or those generated from genetic improvement programs (Wang and van der Kamp, 1992). The fungus induces the premature loss of poplar foliage, altering its photosynthetic capacity and decreasing the vigor of individuals in affected plantations (Frey et al. 2005).

The importance of the damage produced by severe attacks by Melampsora spp. has forced breeding programs for poplars to establish disease resistance as the primary selection objective (Pinon and Frey, 2005; Riemenschneider et al., 2001; Lefèvre et al., 1998; Newcombe et al., 1996; Steenackers et al., 1996). Initially, breeding programs primarily used major breed-specific immunity-granting genes, which were often overtaken by changes in fungal pathogenesis (Pinon and Valadon, 1997; Steenackers et al., 1996; Newcombe, 1996); Steenackers et al., 1994)

The anatomical structures of the poplar are governed genetically and could influence the physiology and morphology of the plant that makes them more or less resistant to the pathogen. The objective of this study was to determine if some anatomical structures of the leaf such as: number of stomata and epithelial cells, leaf area and number of leaves, in addition to the stomatal index, influence the incidence of: *Populus* spp. to the attack of *Melampsora larici-populina*, causal agent of poplar rust in Chile.

MATERIALS AND METHODS

The plant material used was obtained from a Poplar Clonal Conservation Bank of the University of Talca, in the city of Talca, Maule region, Chile (35° 24' S and 71° 38' W). The type of soil in the Bank area is loam, San Rafael series. The annual amounts of precipitation do not exceed 600 millimeters. Average annual temperatures vary between 14° and 19° C, registering maximum temperatures above 30° C during the months of January and February (Dirección Meteorológica de Chile, 2018).

For this study, 50 hybrids from five crosses were used: *Populus trichocarpa* x *P. deltoides* (T x D), *P. deltoides* x *P. trichocarpa* (D x T), *P. deltoides* x *P. nigra* (D x N), (*P. trichocarpa* x *P. deltoides*) x *P. deltoides* (TD x D) and *P. deltoides* (D). The hybrids analyzed had a growth period and were established in a 1.5 x 0.90 meter planting frame and randomly distributed within the nursery, with homogeneous irrigation and fertilization regimes for all the hybrids studied and corresponded to 2 liters. of water / plant / day and weekly fertigation with a solution of macro and micro elements.

Sample collection: The samples were collected at the end of March, at the end of the growth period of the hybrids and when the appearance of the fungus in the area is

greatest. Two clones per hybrid were selected from which two leaves were extracted from the apical, central and basal zone (six leaves in total). The leaves were transferred to a humid chamber, made up of a 20 x 20 cm plastic plate with absorbent paper moistened with distilled water at the bottom and covered with a glass lid, keeping the leaves at high relative humidity. To keep the leaves turgid for a longer time, cotton moistened with distilled water was adhered to the ends of the petioles. These leaves were transferred to a laboratory and used to calculate the number of stomata, epithelial cells, stomatal index, and leaf area.

Stomatal density: To quantify the number of stomata and epidermal cells, the Caldwell and Stone (1932) technique was used. The number of stomata and epidermis cells were counted only on the abaxial face of each poplar leaf and were counted using a Nikon E600 optical microscope with a Nikon coolpix 4300 built-in digital camera, under a field of 40x and 100x.

Stomatal index (IE): the stomatal index was calculated using the formula suggested by Wilkinson (1979):

$$IE = NE * 100 / CE + NE \quad (1)$$

Where: ME corresponds to the number of stomata per field of observation and CE the number of typical epithelial cells in the same field of observation.

Leaf area. The leaves under study were deposited on graph paper and photographed with a Nikon coolpix digital camera at a fixed distance. To calculate the leaf area, each photograph was measured with the J-image version 1.43b program (National Institutes of Health.USA).

Total of leaves: All the leaves were counted for each evaluated plant and the leaves that were affected by rust were also counted to assess the incidence of the disease.

Rust incidence: The field count of the total healthy leaves and leaves attacked by *M. larici-populina* allowed the calculation of the incidence (I) of the disease, using the following formula:

$$I = (HE^* 100) / HT \quad (2)$$

Where: HE correspond to all the leaves that presented symptoms of the diseases and HT correspond to the total leaves present in each plant (hybrid).

Statistical analysis: The variables were statistically processed using the R program, commander version 3.0. An analysis of variance was performed using the HSD Tukey test. The Pearson Correlation coefficient was used to estimate the correlation between rust incidence and the other variables under study, as well as to determine if there is any type of correspondence between them.

RESULTS

STOMATAL DENSITY AND EPITHELIAL CELLS

The calculation of stomatal density per cm^2 (NE/cm^2), stomatal density at the whole leaf level and number of epithelial cells per cm2 (CE/cm^2), showed significant differences at the taxon level ($p = 0.001$). The stomatal density was significantly higher in *Populus trichocarpa* x *P. deltoides*, cross that showed the highest number of stomata per cm2 (22,650 NE/cm2). By contrast, *P. deltoides x P. nigra* presented the lowest number of stomas (16,930 NE/cm2), with a significant difference from the rest of the crosses (Table 1).

Regarding the number of epithelial cells, it was observed that again: *P. trichocarpa x P. deltoides*, presented a greater number of these structures and: *P. deltoides x P. nigra*, a significantly lower amount than the rest of the crosses. The remaining crosses do not present

significant differences in terms of the number of epithelial cells (Table 1).

STOMATAL INDEX, LEAF AREA AND NUMBER OF LEAVES

Regarding the stomatal index, no differences were observed at the level of crosses (p = 0.12) (Table 1). Regarding the leaf area, there were also significant differences between taxa (p=0.11). The cross: *(P. trichocarpa* x *P. deltoides*) x *P. deltoides* presented a leaf area significantly greater than the rest of the taxa (203.7 cm2). The smallest leaf area (129.9 cm2) was observed in the cross: *P. deltoides* x *P. trichocarpa* (Table 1).

In the case of the number of leaves, there are significant differences between the taxa evaluated.

(p= 0,0001). *P. deltoides x P. nigra* presents the largest number of leaves (Table 1).

RELACIÓN DE LAS VARIABLES ANAT CON LA INCIDENCIA DE ROYA

Among the crosses evaluated, it was observed that (*P. trichocarpa* x *P. deltoides*) x *P. deltoides* and *P. deltoides* were not infected with rust, or at least did not develop the disease during this period, being: *P. deltoides* x *P. nigra*, the one with the highest incidence of the disease with 40% infection, followed by: *P. trichocarpa x P. deltoides* and finally *P. deltoides* x *P. trichocarpa* with 36% and 23% rust attack.

A moderate positive correlation (r2= 0.4) was observed between stomatal index and rust incidence, however it is not conclusive since the p=0.051 value is at the limit of significance. A low correlation could be observed for the case of rust incidence and number of stomata per cm2 ($r^2 = 0.14$) and incidence and number of epithelial cells per cm2 ($r^2 = 0.13$). In addition, there was a significant negative correlation between incidence and leaf area ($r^2 = 0.14$; p= 0.0007), in addition to a non-significant negative correlation between leaf area and number of epithelial cells and stomata with (Table 2).

DISCUSSION

The poplars present a wide variation, in terms of their morphological characteristics studied: dimension of their stomata, stomatal density and stomatal index (Orlovic et al., 1998). The differences found between the different crosses in stomatal density, stomatal index and number of epithelial cells on the abaxial surface of the leaf have been confirmed by various authors (Cortan et al., 2017; Radoglou and Jarvis, 1990; Ceulemans et al, 1988). For this study: *P. trichocarpa* x *P. deltoides* presented the highest stomatal density, which agrees with what was discussed by:Ceulemans *et al*, (1984), who shows that some cultivars of *P. trichocarpa* x *P. deltoides* presents a higher stomatal density than: *P. deltoides* x *P. nigra*. In general, the previous results referring to stomatal density, number of epithelial cells, stomatal index and leaf area agree with the results obtained by Pearce et al., (2004); Ferris et al., (2002) and Ceulemans et al., (1984).

The leaf area of each cross presented a negative correlation with the incidence of rust, apparently the greater the leaf area, the lower the disease attack. The latter is in contrast to what was observed in *Salix*, where a positive correlation has been observed between the severity of the rust attack and the leaf area presented by the willows (Toome et al., 2010). However, there are other characteristics of Populus leaves that could influence the apparently opposite results. For example, in those crosses that have larger leaves, they have longer petioles that would allow greater leaf mobility and therefore more difficult the establishment of rust. Other characteristics such as the amount of epicuticular waxes and

	Characteristics of poplar leaves					
taxa	Number of stomata / cm^2 ($*10^1$)	Epithelial cells / cm^2 (10^4)	stomatal index (%)	Leaf area/ cm^2	Total of leaves	rust incidence (%)
(*P trichocarpa* x *P.deltoides*) x *P. deltoides*	1.904 bc	89.1 bc	20.89 a	203,72 a	30 bc	0,0c
P. deltoides	2.107 ab	96.9 ab	20.88 a	171,26 b	28 c	0,0 c
P. deltoides x *P.trichocarpa*	2.056 ab	96.2 ab	20.39 a	153,86 bc	36 ab	23,2 b
P.trichocarpa x *P.deltoides*	2.265 a	107.5 a	19.78 a	138,66 c	33 bc	35,5 ab
P. deltoides x *P. nigra*	1.693 c	78.2 c	21.64 a	129,32 c	40 a	39,9 a

Note: Different letters indicate significant differences according to the Tukey test at 5%.

Table 1: Mean values of stomatal density, epithelial cells, leaf area, number of leaves and rust incidence in five poplar taxa. The values followed by different letters indicate differences according to the HSD Tukey test.

variables	Abaxial epithelial cells	Abaxial number of stomata	Stomatal index	Total of leaves	Leaf area
epithelial cells	1				
stomata	0.56 *	1			
stomatal index	0,52*	-0,29*	1		
total of leaves	0,04 ^{ns}	0,40*	0,03^{ns}	1	
area / leaves	-0,07*	-0,19*	0,02 ^{ns}	-0,08*	1
rust incidence (%)	0,14 *	0,13 *	0,4 *	0,04 ^{ns}	-0,19*

* significant at 5%

ns, not significant

Table 2: Correlations between the number of stomata, epithelial cells, stomatal index, total leaves, leaf area and rust incidence using the Pearson correlation coefficient.

trichomes on the leaves could also influence the results obtained, since leaves with thicker epicuticular wax layers decrease the possibility that rusts can reach the stomatal openings (Alfaro-Tapia et al, 2007; Longo et al., 2006), however, this was not evaluated in the present work.

The results obtained in this study suggest that the number of stomata does not influence the entry of rust uredospores into the poplar leaves, therefore the incidence of the disease is low, which could be due to the fact that the hyphae of the fungus are very small in relation to the size of the stomata (Pei and Shang, 2005). This agrees with what was described by Tian et al. (2002), who observed that several germ tubes can penetrate through the same stoma at the same time. Therefore, a stoma can be the gateway for several spores at the same time, which can quickly invade poplars susceptible to the disease. The other thing that can happen is what Parakash and Heather (1985) suggest, who indicate that the penetration of the fungus into the cell is directly and rarely through the stomata, which is contrary to what was said by Spiers and Hopcroft. (1988) who state that the penetration of the fungus is mainly via stomata and that direct penetration is very scarce. For Yu et al, (2011) direct penetration can also occur. However, the latest works presented by Wan et al., 2015 indicate that the penetration of the germ tubes of: *M. larici-populina* is direct and indirect at the same time. This indicates that the penetration of the fungus into the poplar cell is through its stomata or the mesophyll cells on the abaxial face of the leaf.

On the other hand, the two crosses that presented less susceptibility to the fungus stand out, this agrees with the work of Rubio-Meléndez et al (2011), who states that the cross: (*P. trichocarpa x P.deltoides) x P. deltoides* is less susceptible to attack by Melampsora spp than the cross: *P. trichocarpa x P. nigra,* and that is *Populus nigra,* the one that confers susceptibility to the fungus; the crossing: *P. trichocarpa* x *P. nigra* t also present a higher incidence of the disease. However, this must be evaluated in other studies since the fungus presents several different pathotypes depending on the area of Chile where the poplars are established (Albornoz et al., 2018), which could explain this behavior.

CONCLUSIONS

The anatomical structures of the poplar, such as the number of stomata, number of epithelial cells and number of leaves vary, depending on the taxon to which each of them belongs, as well as the stomatal index and susceptibility to *Melampsora larici-populina.*

A significant mean correlation was observed between the incidence of the disease and the stomatal density, number of epithelial cells, stomatal index and leaf area in the crosses evaluated, suggesting that these structures and the size of the leaves influence the incidence of rust. in the poplars.

The crossings (*P. trichocarpa x P.deltoides) x P. deltoides* y *P. deltoide* were the ones with the highest number of stomata per leaf, the highest stomatal index and the largest leaf area; however, this cross was not attacked by the disease. Being able to indicate that the hybrids of (*P. trichocarpa x P.deltoides) x P. deltoides* have some type of genetic resistance to the fungus.

The crossing: *P. deltoides x P. nigra*, had the lowest stomatal density, the least amount of epithelial cells and the smallest leaf area, however, it presented a higher incidence of rust, which confirms that the number of stomata present in each plant is not related to the incidence presented by poplars. to disease.

The results of this investigation indicate that for the analyzed poplar crosses, the evaluated cell structures, by themselves, are not good indicators as incidence selection

methods at *Melampsora larici-populina*.

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