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"COMPARISON OF METHODS FOR EARLY EVALUATION OF RESISTANCE TO WITCHES´ BROOM *(Crinipellis perniciosa (*Stahel*)* Singer*)* IN COCOA *(Theobroma cacao L.)*".

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ABSTRACT

Three sets of trial were carried out in order to test early evaluation of resistance to *Crinipellis perniciosa*. In all experiments clons SCA 6, SCA 12, CCAT 4675, BE 10 and EET 95 cocoa clones were inoculated with a spore suspension of 1 x 10^4 basidiospores/ml. Two trials dealt with Basidiospores germination on leaf disks and plant extract. Clones showed statistically significance and highly significant for spores germination on leaf disks and water extract respectively. With methods dealing with seedling inoculation disease incidence was 100% and quite severe for all methods: Holliday test, a modified Holliday and the belt spray method. In theses trials, no differences between clones were observe for the incubation period or disease incidence; only the belt spray method gave statistically significant differences for cotyledon persistence on seedlings. In the field inoculation method, the clones showed significant differences for the infection incidence in branches and infection incidence per plant. Basidiospores germination percentage in the leaf disk method and watery extracts method showed a positive correlation with branch infection percentage (0.70 y 0.90) and plant infection percentage (0.90 y 0.70). Cotyledon permanence in the automatic inoculation method was positively correlated with the infection percentage in branches (0.70) and plants (0.60).

BACKGROUND

Different method have been developed and used for early evaluation of Withche´s broom disease in cacao. Some of these as spore germination on leaf disks (Ducamp & Thevenin, 1999), or in aquous extract of young shoots (Evans & Bastos, 1980), the Holliday test (Holliday, 1954), Semiautomatic system of inoculation-infection or "Belt Spray Method" (Frias & Purdy, 1995) and the agar drop method (Evans,) have produced reliable results under different conditions and have been used on WB selection for breeding programs; however differences persists making difficult its extensive use by pathologists and breeders.

This study was design to compare all these methods under similar conditions and with the same cultivars in order to determine which one gives more consistent results under Ecuadorian conditions.

METHODOLOGY

The study was conducted in the Laboratory and experimental fields of the Tropical Experimental Station "Pichilingue" from the Nacional Institute of Agricultural Research (INIAP). The Station is located in the central area of the Coastal plain of Ecuador, 5,5 Km South of Quevedo.

The methods to be evaluated were grouped in three sets of trials, according to the parameter used to evaluate results:

Group 1: spore germination on cocoa leaf disks and on aqueous extract of young shoots.

Group 2: Reaction of young seedlings to inoculation: Holliday test; a modified Holliday test and the Belt spray method.

Group 3: Reaction of plants shoots. Agar drop inoculation of young shoots on the field
Management of the trials.

All methods were tested using open pollinated seedlings and vegetative material of clones: SCA 6, SCA 12, (Resistant); CCAT 4675 (unknown reaction); BE 10 y EET 95 (Susceptible)

Inoculum preparation

Basidiospores were collected from dry broom collected in Pichilingue and placed in broom cabinets to produce sporophores, collected, stored and utilized as recommended by Suárez (1977) and Frias *et al* (1987).

Description of methods

Group 1. Evaluation parameter: spore germination

Trial No. 1 Spore germination on leaf disks (Ducamp & Thevenin, 1999)

Fully developed leaves were collected from the middle of healthy tree branches, they were placed on glass containers with 500 ml of sterile distilled water (SDW) kept on thermal boxes (RUBBERMAID) at approximately 9°C to keep them fresh until taken them to the laboratory. Once there, leaves were washed with SDW and antibacterial soap (PROTEX) and tap dry on paper towels. Disks, 13 mm in diameter were cut with a sacabocado, between the veins of the leaf, they were washed again in SDW and eight disks were placed on Petri dishes lined with sterile filter paper. The paper was wetted with 1ml of SDW. One drop (0,1ml) of spore suspension on water agar (0,3%) was placed on the abaxial surface of each disk. Incubation took place during three hours on lab benches at an average temperature of 23°C.

Trial No. 2. Spore germination on aqueous extract of young cocoa shoots (Evans & Bastos, 1980)

Cocoa shoots were collected early morning and placed on glass flasks with 20 ml of SDW and then maintain in thermal boxes at 9oC to keep them fresh. In the laboratory, shoots were washed with SDW and cut on pieces of about 1 cm. Those pieces were placed in a glass container and then boiling water was pour over them to prepare an infusion (250 ml of water over 2g of leaves pieces). The preparation was cool down in a water bath at 23°C for five minutes. Ten µl of this infusion were placed on each well of a Elisa plate (CORNING 25802). On top of them 100 µl of the spore suspension in water agar (0,3%). Plates were incubated in a humid chamber for three hours on lab benches at an average temperature of 23°C.

In both experiments, once the incubation period was over, germination was stopped applying one drop of Trypan blue on each leaf disk or Elisa well, and then proceed to measure the percentage of spore germination.

Group 2. Symptom evaluation on seedlings

Trial No. 3. Holliday test (Holliday, 1954)

Ripe, open pollinated pods from the selected cocoa clones were harvested & selected for uniformity. The testa was peeled from each seed and then they were placed on plastic basquets with towel paper wetted with SDW. Seeds were germinated in darkness for 4 to 6 days until the radicule was around 2cm in length. Every 48 hours SDW was sprayed over the seeds to keep them wet and favor germination. At this stage, germinated seedlings were rinse with SDW, tap dry with towel paper and submerge during 2 minutes in the spore suspension. Immediately after inoculation, germinated seeds were planted on plastic cones containing 135g of a mixture of soil + peat (BIOLAN[1]). The cones with the plants were kept in plastic racks inside a shade house at ambient temperature (±27 °C).

Trial No 4. Holliday test modified (Suárez, 2004[1])

[1] *Material orgánico en descomposición o apenas ligeramente alterado, acumulado en medio anaerobio. Representa el primer estado en la formación de los carbones. Producto distribuido por Agripac S.A*
[2] *Comunicación personal*

In this case the procedure was exactly as describe for the Holliday test above but instead of submerge the germinated seeds, they were sprayed with a manual sprayer applying 1ml of spore suspension for each seedling.

Trial No 5. Belt Spray Method (Frías, 1987)

Peeled seeds were placed on the cones containing the soil and peat mixture as indicated before, taking care of leaving one fourth of the seed above soil level. Racks with cones were maintained in the shade house for 15 to 21 days until they presented first shoot with primary leaves no longer than 1.5 cm when they were sprayed with a spore suspension in a moving belt as describe by Frias (1987). Inoculated seedlings were places in an incubation chamber for 24 hours. Temperature in the chamber was kept at 27-28 ^{o}C , with 95 -100% relative humidity. Then seedlings in cones were transfer to a shade house until evaluation.

In trials 4 and 5, evaluation parameters were incidence by symptoms(%), incubation period (days) and the time of cotyledons remain attached to the plants.

Trial No 6. Field inoculation

Clonal plants of the selected materials were used. Young branches without lignifications were selected, the apical shoot, pruned so axilar buds become active. These branches were covered with plastic bags to avoid natural infection. Every active bud was inoculated with 0,1 ml of agar spore suspension as the one used in experiments 1 and 2. Following inoculation, branches were again covered with plastic bags (12 x 15cm) but this time a piece of wet paper towel was included to produce a humid chamber to ensure infection. The plastic bag was removed after 7 days.

In this trial parameters measured were percentage of infection per branch and per plant and the incubation period.

Results and Discussion

Group 1. Trials 1 and 2. Spore germination on two different substrates

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Results obtained on the two trials can be seen on table 1. Spore germination on leaves was very low, ranging from 5 to 7%. However the Chi^2 test showed significant difference between clones, with the resistant SCA 6 having the lowest value (4,83) and the CCAT 4675 having the largest percentage (7,91). It is well known that *M. perniciosa* prefer very young, meristematic tissue, therefore although the leaves used in this trial were still tender, they were fully grown and may have some inhibitor that prevented the fungus to germinate. It is not discarded as well the possibility stated by Brownlee et at (1993) about the presence of tannins on leaves that may act against the fungus.

Table 1.- ***C perniciosa*** spore germination on two different substrates from five cocoa clones (***Theobroma cacao*** **L.**). INIAP. Pichilingue. Quevedo-Los Ríos, 2005.

Clon	Sustrates (mean values)	
	Leaves disks *	Aquous extracts
	Chi^2: 10.67 *	Chi^2: 18.43 **
SCA 6	4.83	88.64
SCA 12	4.87	89.89
CCAT 4675	7.91	92.58
BE 10	5.58	95.05
EET 95	5.19	95.39
Control +	-	95.86

*Chi2, Kruskal-Wallis Test, *p=0,01 and **p=0,001*
+ Control: Water + sporas), Value not considered on the análisis.

On the other hand, as may be expected on aqueous extract of young shoots, percentage of germination was comparable to that in pure water (Evans y Bastos, 1980). Again, although numerical differences are very short, germination on extracts from SCA6 and SCA12 presented some factor that reduced germination significativally (88.64 %, and 89,89% respectively). Various factors may have influence the percentage of germination, one could be a high dilution of the content of the extract that prevent larger differences between clones; the use of Tween-80 as dispersant agent as well may have prevented tannins which, as in the leaves, could have prevented the development of the germ tubes. In the two experiments, the SCA reaction was as expected in a resistant material. The reaction of the other three clones is variable, especially for CCAT 4675 and EET 95 that presented opposite ranking in both trials, however differences are so short that is not possible to reach clear conclusions. The clon BE10 kept same ranking in both trials, being on the susceptible side.

Group 2. Symptom evaluation on seedlings

This group of trials included those methods based on the reaction of seedlings to *M. perniciosa*: Holliday test, H. test modified and the belt spray method.

Seedlings presented a range of symptoms as describe elsewhere for Witches broom infection (Table 2). Controls were symptomless, stem roughness (RT) and cotiledonary brooms (EC) were the most frequent symptoms, followed by hypersensitive reaction (RH) that kill inoculated seedlings.

Table 2.- Percentage of incidence and Number of plants with different types of symptoms with three methods to evaluate Incidencia de los diferentes síntomas iniciales de ***C. perniciosa*** en tres métodos de evaluación temprana. INIAP. Pichilingue. Quevedo-Los Ríos.2005.

Methods	Clones	No. of inoculated seedlings	Number of plants with symptoms								Infectión %
			HH	HNC	HE	AH	RT	EC	ET	RH	
Holliday Test	SCA 6	103	21	19	13	7	73	23	0	35	100
	SCA 12	139	22	8	28	18	68	35	1	8	100
	CCAT 4675	116	29	29	29	0	66	51	3	10	100
	BE 10	91	23	14	13	6	52	29	0	24	100
	EET 95	172	38	26	66	4	73	83	2	12	100
	Testigo	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Holliday test modified	SCA 6	77	11	8	1	13	58	28	0	43	100
	SCA 12	123	8	8	7	16	69	29	0	8	100
	CCAT 4675	124	27	22	5	2	64	69	0	15	100
	BE 10	82	20	17	2	2	59	27	0	26	100
	EET 95	177	45	35	31	5	108	89	1	29	100
	Testigo	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Belt spray system (SAI)	SCA 6	73	0	0	10	0	61	29	7	21	100
	SCA 12	153	1	4	17	0	117	61	22	33	100
	CCAT 4675	82	0	1	6	0	62	27	5	16	100
	BE 10	57	0	0	16	0	51	8	15	6	100
	EET 95	148	0	12	12	0	102	73	12	55	100
	Testigo:	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**

HH: *Hypocotile swollennes,* ***HNC:*** *Coteledonal node swollen,* ***HE:*** *Swollen Epicotile,* ***AH:*** *Ahilamiento of stem,* ***RT:*** *Stem roughgenness,* ***EC:*** *Cotiledonal broom,* ***ET:*** *Terminal broom,* ***RH:*** *Hypersensitive reaction*

The total numbers of symptoms override the number of plants because many seedlings presented more than one symptom. The relatively high incidence of the hypersensitive reaction on these trials indicates high inoculums pressure. This same factor plus the virulence of the Ecuadorian strain, may account for

the high disease incidence (100%) on all treatments that did not allow discriminating levels of susceptibility (Frias, 1995).

The incubation period was as well very similar (Table 3) in all treatments and differences found in Brazil by Andebrhan et al (1998) and others on this parameter was not observed under the conditions of this experiments. Concentration of the spore suspension and the age of the seedlings may require adjustments to the aggressiveness of the Ecuadorian strain of *M perniciosa*.

Cuadro 3: Incubation period of ***M. perniciosa*** in days for three methods of Early evaluation of resistance for five cocoa clones. INIAP. Pichilingue, 2005.

CLON	Inoculation Methods		
	Holliday	Holliday Modified	Belt Spray
	Chi^2: 2.70 ^{ns}	Chi^2: 2.30 ^{ns}	Chi^2: 2.27 ^{ns}
SCA 6	17.86	17.71	18.39
SCA 12	16.60	17.75	19.43
CCAT 4675	17.25	18.34	18.55
BE 10	18.80	17.52	19.79
EET 95	16.34	17.14	17.72

** Mean values. ns=no significance for Chi^2 Test from Kruskal-Wallis*

In these studies, special attention was put on measuring for the first time the permanence of cotyledons on inoculated plants as compared with non inoculated. Although Holliday (1955) mentioned the non-abscission of cotyledons in artificially inoculated seedlings, it has not been considered a discriminator system and is not describe as such on the detailed symptoms descriptions published (Baker and Holliday,1957 and Rudgard, 1989). Non-inoculated plants allowed abscission of cotyledons between 34 and 54 days, so we can speculate that it is a factor that depends on the genetic constitution of the clones; however cotyledons on the inoculated seedlings remain on the plants up to 84 days (Table 4). Both Holliday tests showed no differences between clones for this parameter, but the belt spray method gave statistically significant differences between clones, in agreement to the statement of Motilal *et al* (2003); with the SCA12 and 6 in one end with the lower value (0,20 and 8 days difference respectively with their controls); and the susceptible EET95, the highest (22 days of difference from the control).

CUADRO 4: Permanence of cotyledons in days after infection for three early evaluation methods for resistance against Witches Broom in cacao seedlings.

CLON	Inoculation methods					
	Holliday	Control	Holliday Modificado	Control	SAI	Control
	Chi^2: 1.73 ns		Chi^2: 2.88 ns		Chi^2: 18.97 **	
SCA 6	84.40	45.74	68.38	45.74	42.67	33.79
SCA 12	81.31	47.60	78.14	47.60	41.36	41.16
CCAT 4675	81.84	39.45	79.34	39.45	54.69	42.24
BE 10	69.92	48.57	84.20	48.57	49.36	37.30
EET 95	80.74	54.94	74.94	54.94	70.48	47.88

** Valores Medios. Abreviatura ns muestra la no significancia en la prueba del Chi^2 del test de Kruskal-Wallis.*

Group 3. Field inoculations

Despite that this test gave very low values, differences were highly significant between clones (Table 5), both for disease incidence and for incubation period. Low infection may be a consequence of

predominant weather conditions during this trial, with high light intensity and low relative humidity (low rain) which could be affected the fungus and the branches as well. However it may well be interesting to take these factors into account and consider these variables for early tests as has been proposed by Almeida *et at* (1991).

Cuadro 5: Percentage of infection per branch and per plant and incubation period on young shoots for five cocoa clones (*Theobroma cacao* L.) inoculated in the field by the agar drop method.

	Disease incidence %		Incubation period days
CLON	Per branch	Per plant	Young shoots
	Chi^2 : 10.64 *	Chi^2 : 15.33 **	Chi^2 : 9.95 **
SCA 6	0.45	0.16	71.00
SCA 12	3.16	2.07	42.39
CCAT 4675	3.17	4.11	45.38
BE 10	4.91	5.09	49.04
EET 95	3.40	3.59	44.63

*Mean values: * p: 0.01; ** p: 0.001 for Chi^2, Kruskal-Wallis Test.*

Correlation between the evaluation methods

Attempts were done to correlate variables of the different methods evaluated, finding interesting results: the variable percentage of germination in both methods used had high correlation with percentages of branch and plant infection (0.90 and 0.70 respectively) in the field. However, it is not recommended to use methods that require counting of spores to evaluate large number of materials, because it is a very tedious and time consuming procedure, in the same line, was very difficult to find adequate material in the field to collect shoots to prepare the extract, and this is going to handicap the use of this type of method in large scale.

In the case of the Belt spray method, the variable permanence of cotyledons was as well highly correlated with branch (0.7) and plant (0,6) infection in the field. This method as stated by Frias (1987, 1995), and Purdy et at (1998) is showing the most promising possibilities because once the facilities are set up in place, the procedure allow handling of large amounts of plants, and reproduce infection consistently. It is necessary however to adjust the concentration of the inoculums and the age (or rather the stage) of the shoot to inoculate, factors that are so far hampering this method.

The inoculation of shoots at the field presented as well interesting possibilities, but is too dependent on selecting adequate material on the field, and we can estimate that there would always be weather factors altering the reaction of cocoa plants to artificial inoculation at this level.

CONCLUSIONS

- The reaction of the plants in the five methods compared were of a magnitude that did not allow proper discrimination between the clones;
- Variables to measure vary with the method;
- The permanence of cotyledons in the belt spray method; spore germination on both methods used, and field infection were the variables and methods that allowed to appreciate differences.
- Although differences found with the different methods were very short, clones SCA, 6 y 12 did showed its resistant conditions and the EET 95 maintained as well its susceptible condition.
- Efforts should be addressed to overcome factors and conditions that affect the efficiency of the methods;
- A "calibration" of the stage of the substrate to use and the spore concentration seems to be a condition to fulfill wherever and whenever one of this methods will be used.

- The modified Holliday test, the belt spray and the agar drop methods can be the tool breeders and pathologist require for WB resistant material selection, once the adjustments sugested in this comparative work be completed.

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