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An efficient method for selecting stable tester strains of vegetative compatibility groups in V*erticillium dahliae*

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Abstract: The way compatible pairs of nitrate non-utilizing mutants (*nit*) are selected is usually not explained and remains unclear whether these pairs are representative for an isolate or strain. In addition, tester strains of *Verticillium dahliae* vegetative compatibility groups (VCGs) cross-react with at least one pair of tester strains of another VCG, and although it is a common knowledge of scientists working with the fungus that reversion of nit mutants to wild type occurs far too often, this fact is rarely mentioned in papers. To overcome the above problems, a protocol was developed for the generation of large number of nit mutants from any given isolate and to ensure that compatible pairs of mutants are indeed stable and the most frequent within the putative tester mutants produced from each isolate. Thus, we provide a reproducible and objective way of selecting *V. dahliae* tester strains for each isolate and VCG. Although VC grouping is based on the formation of stable heterokaryons, we demonstrate in this work that cross-reactions cannot be eliminated and that strict genetic barriers between two main VCG groups are absent in *V. dahliae*.

Key words: efficient heterokaryon formation as a criterion, group testers, isolate testers, vegetative compatibility, V*erticillium dahliae*

Introduction

Almost all published reports on the formation of *Verticillium dahliae nit* mutants that are grouped in Vegetative Compatibility Groups (VCGs) are based on only two selection criteria: (a) phenotypes of nitrate non-utilizing mutants *nit* M and *nit* 1 and (b) strong heterokaryon formation, resembling wild type – after pairing these mutants (Strausbaugh 1993; Zeise and Tiedemann 2001; Dervis 2007).

However, it is not presented how the pairs of compatible tester strains are chosen, what is the stability of these mutants in continuous subculturings and whether or not these selected strains are representative for best performance amongst many mutants derived from the same isolate. Moreover, we noted the high reversion rate of *nit* M mutants. These observations prompted us to readdress the question of how reliable are the VCG approaches we use and how we can guarantee that the strains used as testers are indeed stable and the most representative of each isolate.

In most works on VCGs there were only two criteria based on which the pairs of compatible mutants were selected: phenotypes *nit* M and *nit* 1 and strong heterokaryon formed after pairing of these mutants. Strong reaction always resulted in the formation of heterokaryons resembling the wild type of the fungus. So far in most works only such single pairs of mutants were used for the assignment of isolates to VCGs. In so many works no more information concerning the selection of compatible pairs is given. We do not know how the pair of chosen mutants is representative for the isolate. Have the mutants been chosen in the arbitrary way or on the base of additional objective criteria?

Many times, in most cases *nit* M mutants reverted, often after heterokaryons have been formed. A possibility to eliminate this phenomenon was not exploited.

In our studies on vegetative compatibility in *V. dahliae* we have observed bridges between American testers VCG 2 and VCG 4 (Joaquim and Rowe 1990), also between American and Duch testers. We got heterokaryons between VCG 1 and Dutch NL II (corresponding to VCG 2), between subgroup testers VCG 2A and 2B, VCG 4A and 4B (Hiemstra and Rataj-Guranowska 2003; Rataj-Guranowska 2006) VCG 6 and Dutch NL II (personal observation). Also other researchers noted cross-reactions between American testers, however not between so many of them (Elena and Paplomatas 1998; El-Bebany *et al*. 2013; Papaiannou and Typas 2015). Such a situation hinders understanding of genetic relationship between newly characterized isolates and existing VCG testers. Considering these observations we tried to check if there is possibility to eliminate so many of cross-reactions.

The aim of the work was to elaborate the procedure to recover many *nit* mutants, to select the pairs of mutants forming heterokaryons most frequently within the group of mutants, which were recovered from each isolate and to eliminate the reverted mutants.

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There are two ways to perform the modified test: the systematic, more laborious and the quick one approachable in the range of experienced workers.

Materials and Methods

Isolates of *V. dahliae* and Dutch testers of VSGs from the work of Hiemstra and Rataj-Guranowska (2003) are cited as an example. When the group of the isolates originates from a new plant or new localization, about 30 isolates are the minimum number to be characterized.

VCGs determination in a systematic way

Recovery of nit mutants

Like in other studies on vegetative compatibility single-spored cultures were employed. The isolates of *V. dahliae* were grown on Potato Dextrose Agar (PDA, Difco) and Carrot Medium (CM) (Hiemstra and Rataj-Guranowska 2003), amended with 6% of chlorate. Each single-spored culture was subcultered at four points on agar in Petri dish (10-cm of diameter). After 7–10 days the presumed mutants appeared and they were taken from inner parts of colonies, where the concentration of chlorate was even higher than 6% and subcultered at 21 points (Fig. 1) in Petri dishes with Minimal Medium (MM) (Correll *et al*. 1987) – two dishes per each medium. After following 7–10 days stable *nit* mutants were phenotyped. Usually about 30–60 mutants appeared. They were inoculated on MM and the hypoxanthine medium (Correll *et al.* 1987). The first set of MM plates were kept for 21 days and observed to eliminate reverting mutants.

Phenotyping of nit mutants

The *nit* mutants were classified as *nit* 1, and *nit* M based on the differences in their utilization patterns of the nitrogen source in the medium (Correll *et al.* 1987).

Complementation, vegetative compatibility

All the *nit* M mutants (up-to 9–12 in most cases) and 3 *nit* 1 mutants from each isolate were paired with each other. It was arranged at 15 points in Petri dishes (of 10-cm diameter) (Fig. 1). The results of pairing were scored after 7, 14 and 21 days. Pair (two pairs) of mutants which were the most frequent within the putative tester mutants produced from each isolate (Fig. 2; Table 1) were called the strain testers and transferred to 5-cm diameter Petri dishes with MM medium.

Pairs of the strain testers were paired with each other and VCGs were designated. Then the VCGs testers were paired with the set of American VCG testers: V44 (VCG 1), PH, 115 (VCG 2a, VCG 2B), PCW (VCG 3), BB, S39 (VCG 4A, 4B) and Dutch testers (NL I, NL II).

Determination of the group testers

All the strain testers were paired with themselves and VCGs were determined. After checking about 30 isolates VCGs were usually assumed and VCG testers as well. The following isolates, starting from 31 were paired with the VCG testers.

However, our very careful selection of the isolates and group testers is time-consuming. With the growing experience the quick procedure was elaborated. This procedure, however, is approachable in the range of experienced workers.

Quick method of VCG determination

Inoculation, incubation and recovery of *nit* mutants were performed as described above for the systematic procedure.

The presumed mutants were subcultered only on MM medium. The Petri dishes were observed for at least three weeks. We looked for heterokaryons appearing in acci-

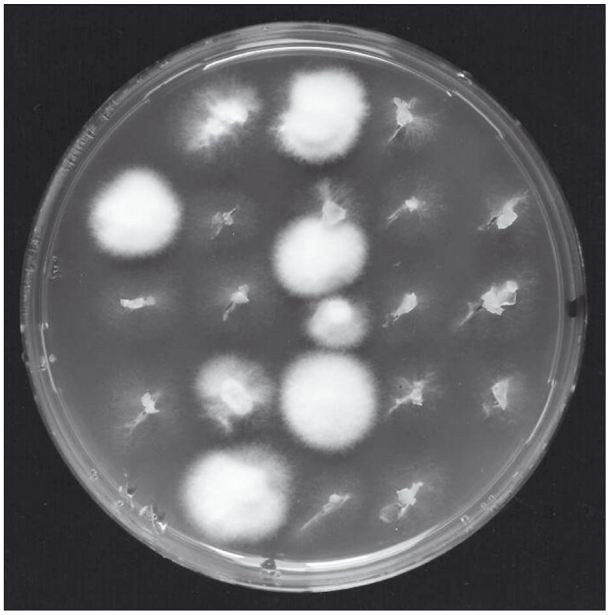

Fig. 1. *Nit* mutants subcultured on Minimal Medium (MM) at 21 points together with several wild isolates and two mutants reverting

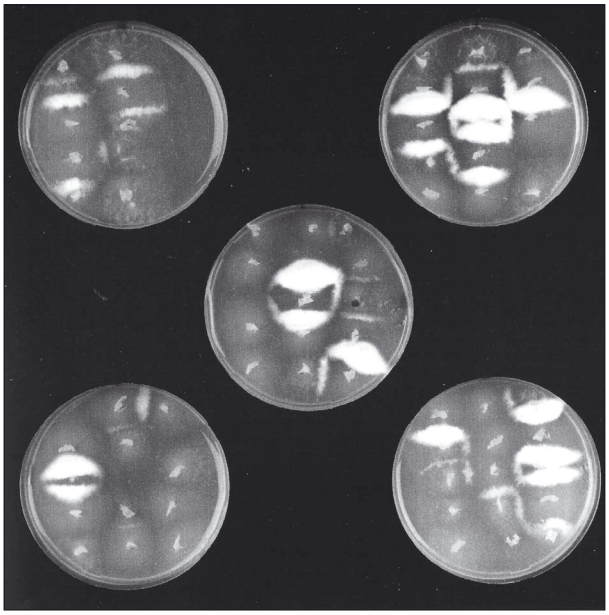

Fig. 2. Pairing of *nit* mutants subcultured on Minimal Medium (MM)

Table 1. Pairing of *nit* mutants within the isolate No. 11 of *Verticillium dahliae*

	nit 1			*nit* M						
	2	10	30	5	19	20	21	22	23	31
nit 1										
2	nt									
10	–	nt								
30	–	–	nt							
nit M										
5	–	–	–	nt						
19	–	–	–	–	nt					
20	–	++	++	++	–	nt				
21	++	++	++	++	+/–	++	nt			
22	+/–	–	–	–	+/–	++	++	nt		
23	–	–	+/–	–	–	–	–	–	nt	
31	++	++	+/–	++	+/–	++	–	+/–	–	nt
No. of compatible pairs										
	nit 1/*nit* M					*nit* M/*nit* M				
	3	3	4	3	3	4	4	4	0	5

The isolate testers of isolate No. 11: *nit* M 31/*nit* M 20 and *nit*1 10/*nit* M 21
"nt" complementation not tested, "++" very strong complementation, "+" weak complementation, "+/–" very weak complementation, "–" no complementation

dental combinations of mutants subcultured at 21points on Petri dish (Fig. 3). They were named the heterokaryons *in situ*. Having at least four dishes and a lot of mutants it was possible to get stable and compatible pairs of mutants. The reverted mutants could be removed. Sometimes it was possible to get enough pairs on one Petri dish (Fig. 3). The compatible pairs were paired with themselves in the way written above. Only selected two pairs of strain testers were phenotyped, as above.

The whole procedure is much shorter than in the systematic way. Still the modification needs an experienced and skilful person.

Fig. 3. Heterokaryons formed *in situ* in many accidental arrangements of *nit* mutants. Some of mutants reverting to the wild state after formation of heterokaryons

Results and Discussion

By means of our modified method we were able to recover many *nit* mutants: 30–60, not one pair from each isolate. We got often almost 100% mutants from 21 subcultures on the dish. We exploited mainly *nit* M mutants, genetically the most differentiated group of mutants. The pairs of *nit* M/*nit* M were the most efficient in heterokaryon formation, in many cases better than the pairs of *nit* M/*nit* 1 (Hiemstra and Rataj-Guranowska 2003).

In most studies of VCGs determination the criterion based on which the VCG testers were chosen is not known. The only information given claims that the pairs of *nit* M/*nit* 1 form quickly strong heterokaryon (Correll *et al.* 1987). In our modified procedure the selection of most frequent pair of each isolate was very helpful. As it is shown on figure 2, in case of isolate 11 there were many of very strong heterokaryons forming in different combinations of mutants. It is not possible to make choice of the best pair of testers just looking at the heterokaryons. Our procedure presented here is based on the sound criterion – the number of heterokaryons formed.

The presented procedure provides very careful and objective way of selection of stable and representative, compatible pairs of mutants before complementation test is performed. This procedure might be applied in other pathogenic fungi, for example *Fusarium oxysporum.* However, the modified method might give different results in VCG assignment, especially of groups VCG 1, VCG 2, and VCG 6 (Rataj-Guranowska and Lukaszewska-Skrzypniak 2010) than by application of standard method. Papaioannou and Typas (2015) mentioned such a possibility.

However, applying such a careful procedure, the cross-reactions could not be eliminated in *V. dahliae*. In the study of Hiemstra and Rataj-Guranowska (2003) within 44 isolates studied one case of bridge-isolate happened.

Two Dutch group testers NL I and NL II formed strong heterokaryons with the isolate No. 6. The heterokaryon was stronger with NL II. The Dutch testers of two main vegetative compatibility groups in *V. dahliae* did not make heterokaryons with themselves, as did American testers of these groups.

This result confirms the conclusions of Papaioannou and Typas (2015) about the absence of strict genetic barriers between VCGs in *V. dahliae*. According to the authors a spectrum between highly stable and more unstable heterokaryosis exists in *V. dahliae.* There is no clear cut compatibility or incompatibility. In our works the continuous spectrum between VCGs and NLs also exists.

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