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Investigation of *nit* mutant generation in Iranian isolates of *Valsa sordida*

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Species of *Valsa* are common pathogens of woody plants all over the world. Generation of nitrate non-utilizing mutants was investigated in fourty two, six and one isolate of *V. sordida*, *Cytospora* sp.1 and *Valsa* sp.1 respectively. These were obtained from different host plants grown in diverse areas of Iran. Minimal medium (MMC), Czapek-dox agar (CZC) and MM+rose Bengal amended with 4.5% to 6% potassium chlorate were used to generate *nit* mutants. Of the forty-nine isolates, *nit* mutants in 29 isolates were recovered on MMC+4.5% $KClO_3$ and in 14 isolates on MMC+6% $KClO_3$. For three isolates, *nit* mutants could not be obtained after enhancing the potassium chlorate to 6% and three other isolates did not grow on MMC. None of the fungal isolates were able to grow on CZC and MMC+rose Bengal. The phenotype of all generated *nit* mutants was identified as *nit3* indicating mutation in *nit3* locus of the isolates that affected the pathway-specific regulatory protein. No heterokaryon was observed in pairings of identified *nit* mutants in complementation tests on MM containing $NaNO_3$**.** This study is the first to investigate generation of *nit* mutants, which is a first step for distinguishing vegetative compatibility groups in *V. sordida* isolates.

Key words: Canker, *Cytospora*, Fungi, Nitrogen metabolism, *Nit* mutants and VCG.

Introduction

Cytospora canker which is sometimes referred to as Valsa canker or Leucostoma canker is caused by species of the genus *Cytospora* Ehrenb., and their related teleomorphs; *Valsa* and *Leucostoma*, which is a worldwide problem and affects woody shrubs and trees (Adams *et al*., 2006; Mehrabi *et al*., 2011). In general, *Valsa sordida* and its anamorph, *Cytospora chrysosperma*, were identified as the causal agent of the canker disease on species of *Populus* and *Salix* and more rarely on other angiosperms in all over the world (Hayova and Minter, 1998). Recently, Fotouhifar *et al*. (2010) identified many other host plants for *V. sordida* in Iran, indicating the expanding host range of the species.

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Aghapour (2010) investigated the morphological characteristics and genetic diversity of *Valsa sordida* isolates, the causal agent of tree canker diseases in Golestan province of Iran, using rep-PCR molecular marker. According to the results, the fungal isolates occupying the cankers were separated into three taxa, *V. sordida*, *Valsa* sp.1 and *Cytospora* sp.1 based on morphological and molecular aspects.

Abbasi *et al*. (2011) estimated the extent of genetic diversity in *C. chrysosperma* isolates collected from different agro-climatic walnut tree growing regions of Iran using RAPD primers. Based on the results, isolates were divided into six groups, showing a high genetic diversity in the population of *C. chrysosperma*. There was no correlation between geographic origins of the isolates and the identified groups in molecular investigation.

Vegetative compatibility by generation of *nit* mutants has been widely documented among several fungi and has proved to be a powerful tool in determining fungal genetic diversity (Puhalla, 1985; Correll *et al*., 1987, 1988; Nitzan *et al*., 2002). Determination of vegetative compatibility groups (VCGs) is a way to study genetic variability in fungal populations and at the first time was employed by Puhalla (1985) to distinguish and classify strains of *Fusarium oxysporum*. During the vegetative compatibility, heterokaryon formation is a complex phenomenon that depends on more than just the ability of strains to complement physiologically (Leslie and Summerell, 2006).

Vegetative compatibility is genetically regulated, and two isolates are considered to be in the same group, if they anastomose and form stable heterokaryons (Leslie, 1993). Therefore, isolates belonging to a same VCG may have similar pathological and physiological traits if they accurately correlate with the results of pathogenicity and other physiological tests (Leslie, 1993; Gichuru *et al*., 2000; Aqeel *et al*., 2008). In asexual fungi, isolates in one VCG cannot exchange genetic material with those inside the other VCGs and are believed to be most similar genetically. However, Masel and colleagues in 1996 reported some evidence of inter-VCG exchange of genetic material (Gichuru *et al*., 2000). In different fungi, varying degrees of success have been achieved in using VCG to characterize isolates into pathotypes or geographic origins (Correll *et al*., 1993; Gichuru *et al*., 2000). In general, vegetative compatibility provides a basis for distinguishing isolates within a species, and has been used extensively to characterize fungal populations (Leslie, 1993). However, vegetative compatibility groups do not always show a clear association with genetic individuals, as is exemplified by a study of population of *Sclerotinia sclerotiorum* on *Ranunculus ficaria*, in which DNA fingerprint and vegetative compatibility group affiliation were found to be decoupled (Kohn, 1995). Mohammadi and Mofrad (2010) investigated the genetic

diversity of *Fusarium verticillioides* isolates obtained from corn by determination of vegetative compatibility groups and pathogenicity test. Based on the results, there was no correlation between geographic distribution, pathogenicity and determined VCG groups.

In order to characterize and classify fungal isolates according to VCGs, one possibility is to develop strains that are both resistant to chlorate and unable to use nitrate as the sole source of nitrogen, the so-called "nitrate non-utilizing" or *nit* mutants (Barcelos *et al*., 2011). The genetic system regulating VCG is encoded by nuclear genes in certain fungal species. Vegetative compatibility occurs only between closely related genotypes, and numerous grouping imply numerous genetically different individuals (Wang *et al*., 1998).

Jensen and Adams (1995) studied the nitrogen utilization pattern of two species of *Leucostoma* using virulent isolates of *L. persoonii* and *L. cincta*, and two virus-infected hypovirulent isolates of *L. persoonii*. The ability of isolates to utilize nitrogen sources was different. Some were able to utilize most of nitrogen sources, but other isolates were unable. Inability to utilize specific nitrogen sources is likely the result of mycovirus infection. According to their results, poor growth of some isolates on several utilizable nitrogen sources might be influential in hypovirulence.

Helton and Konicek (1962) studied on optimum environment for the culture of *Cytospora cincta* and *Cytospora leucostoma* isolates. The experiment was conducted to determine the effects of six common nitrogen-containing compounds on growth and sporulation. The quantitative-growth experiments demonstrated that, in general, *Cytospora* species have rather flexible abilities to utilize different sources of nitrogen.

The objectives of the study were to develop *nit* mutants and to use the *nit* mutant system to characterize the genetic diversity of *V. sordida* populations that were obtained from different geographic regions and from various host plants in Iran.

Materials and methods

Fungal isolates

Most of the *V. sordida* isolates used in this study were obtained from the studies done in the past by Aghapour (2010) and Fotouhifar *et al*. (2010) which were collected during the years, 2004 to 2008. Few additional isolates infecting twigs of trees with Cytospora canker symptoms and having sexual or asexual fruiting bodies were collected from the Semirum region of the Isfahan province of Iran during the year 2010. After the morphological identification of the fungi accompanying the canker symptoms, *V. sordida* isolates were recovered

through the single spore subcultures using conidial masses or ascospores as described by Adams *et al*. (2006). All pure fungal isolates were stored in filter paper at -20 °Ċ. Forty-nine isolates were selected for VCG determination. The geographic and host origin of the isolate is shown in Table 1. All studied specimens were kept in the herbarium of Department of Plant Protection, University of Tehran, Karaj, Iran, and will be deposited in the Herbarium of Iran (Iranian Research Institute of Plant Protection, Tehran, Iran) when the study is finished.

Table 1. Examined isolates of *V. sordida* (anamorph; *C. chrysosperma*), *Valsa* sp.1 and *Cytospora* sp.1

Isolate name	Taxon	Host	Geographic origin	Year	Collector	No. of screened mutants
G107-T	*Valsa* sp.1	*Populus deltoides*	Golestan	2008	B. Aghapour	20
G54	*Cytospora* sp.1	*Populus deltoides*	Golestan	2008	B. Aghapour	20
G72	*Cytospora* sp.1	*Populus deltoides*	Golestan	2008	B. Aghapour	14
G94	*Cytospora* sp.1	*Populus deltoides*	Golestan	2008	B. Aghapour	20
G105-yell	*Cytospora* sp.1	*Populus deltoides*	Golestan	2008	B. Aghapour	20
G107	*Cytospora* sp.1	*Populus deltoides*	Golestan	2008	B. Aghapour	0
G116	*Cytospora* sp.1	*Populus deltoides*	Golestan	2008	B. Aghapour	5
G32-T	*Valsa sordida*	*Salix babylonica*	Golestan	2008	B. Aghapour	41
G38	*Cytospora chrysosperma*	*Salix triandra*	Golestan	2008	B. Aghapour	17
G61	*Cytospora chrysosperma*	*Salix fedtschenkoi*	Golestan	2008	B. Aghapour	52
G78	*Cytospora chrysosperma*	*Salix babylonica*	Golestan	2008	B. Aghapour	14
G104	*Cytospora chrysosperma*	*Salix babylonica*	Golestan	2008	B. Aghapour	27
G105-red	*Cytospora chrysosperma*	*Populus deltoides*	Golestan	2008	B. Aghapour	0
G108	*Cytospora chrysosperma*	*Populus nigra*	Golestan	2008	B. Aghapour	26
G109	*Cytospora chrysosperma*	*Populus deltoides*	Golestan	2008	B. Aghapour	20
G95	*Cytospora chrysosperma*	*Eucalyptus* sp.	Golestan	2008	B. Aghapour	13
G91	*Cytospora chrysosperma*	*Populus nigra*	Golestan	2008	B. Aghapour	0
102	*Cytospora chrysosperma*	*Populus alba*	Chaharmahal	2004	Kh.-B. Fotouhifar	16
103	*Cytospora chrysosperma*	*Platanus orientalis*	Tehran	2004	Kh.-B. Fotouhifar	0
109	*Cytospora chrysosperma*	*Platanus orientalis*	Tehran	2004	Kh.-B. Fotouhifar	35
184	*Cytospora chrysosperma*	*Salix* sp.	Fars	2005	Kh.-B. Fotouhifar	13
200	*Cytospora chrysosperma*	*Fraxinus excelsior*	Markazi	2005	Kh.-B. Fotouhifar	56
203	*Cytospora chrysosperma*	*Fraxinus excelsior*	Markazi	2005	Kh.-B. Fotouhifar	40
215	*Cytospora*	*Ligustrum*	Lorestan	2005	Kh.-B.	22

	chrysosperma	*latifolium*			Fotouhifar	
225	*Cytospora chrysosperma*	*Robinia pseudoacacia*	Lorestan	2005	Kh.-B. Fotouhifar	6
226	*Cytospora chrysosperma*	*Armeniaca vulgaris*	Lorestan	2005	Kh.-B. Fotouhifar	27
268	*Cytospora chrysosperma*	*Salix excelsior*	Kurdistan	2005	Kh.-B. Fotouhifar	38
282	*Cytospora chrysosperma*	*Crataegus azarolus*	Kurdistan	2005	Kh.-B. Fotouhifar	9
300	*Cytospora chrysosperma*	*Morus alba*	Hamadan	2005	Kh.-B. Fotouhifar	4
304	*Cytospora chrysosperma*	*Malus pumila*	Hamadan	2005	Kh.-B. Fotouhifar	37
305	*Cytospora chrysosperma*	*Tamarix* sp.	Qazvin	2005	Kh.-B. Fotouhifar	35
308	*Cytospora chrysosperma*	*Populus nigra*	Semnan	2005	Kh.-B. Fotouhifar	20
321	*Cytospora chrysosperma*	*Platanus orientalis*	Razavi Khorasan	2005	Kh.-B. Fotouhifar	23
331	*Cytospora chrysosperma*	*Olea sativa*	Mazandaran	2005	Kh.-B. Fotouhifar	20
335	*Cytospora chrysosperma*	*Juglans regia*	Tehran	2005	Kh.-B. Fotouhifar	0
150-1	*Cytospora chrysosperma*	*Prunus domestica*	Kohkiluyeh	2005	Kh.-B. Fotouhifar	10
284-1	*Cytospora chrysosperma*	*Thuja orientalis*	Kurdistan	2005	Kh.-B. Fotouhifar	3
317-1	*Cytospora chrysosperma*	*Thuja orientalis*	Razavi Khorasan	2005	Kh.-B. Fotouhifar	21
54-1	*Cytospora chrysosperma*	*Salix aegyptiaca*	West Azarbaijan	2004	Kh.-B. Fotouhifar	26
69-1	*Cytospora chrysosperma*	*Persica vulgaris*	West Azarbaijan	2004	Kh.-B. Fotouhifar	30
69-2	*Cytospora chrysosperma*	*Persica vulgaris*	West Azarbaijan	2004	Kh.-B. Fotouhifar	0
279-1	*Cytospora chrysosperma*	*Juglans regia*	Kurdistan	2005	Kh.-B. Fotouhifar	20
279	*Valsa sordida*	*Juglans regia*	Hamadan	2005	Kh.-B. Fotouhifar	25
S 3	*Cytospora chrysosperma*	*Populus deltoides*	Esfahan	2010	S. Bozorgmanesh	35
S 9	*Cytospora chrysosperma*	*Populus deltoides*	Esfahan	2010	S. Bozorgmanesh	6
S 14	*Cytospora chrysosperma*	*Salix babylonica*	Esfahan	2010	S. Bozorgmanesh	20
S 16	*Cytospora chrysosperma*	*Salix fedtschenkoi*	Esfahan	2010	S. Bozorgmanesh	26
S 24	*Cytospora chrysosperma*	*Juglans regia*	Esfahan	2010	S. Bozorgmanesh	6
S 25	*Cytospora chrysosperma*	*Elaeagnus angustifolia*	Esfahan	2010	S. Bozorgmanesh	6

Isolation and characterization of nit mutants

In an initial test, chlorate-resistant mutants were generated on minimal agar medium containing potassium chlorate (MMC) (Correll *et al*., 1987), MMC containing 50 mg/l rose Bengal (10 ml of 5 mg/ml stock solution in 95 % ethanol) (Elias and Cotty, 1995), and Czapek-Dox agar medium containing

potassium chlorate (CZC) (Correll *et al*., 1987). Minimal medium (MM) was prepared by adding 2 grams of $NaNo_3$ and 30 grams of sucrose to 1 liter basal medium (Correll *et al*., 1987). MMC was prepared by adding 1.6 grams of L-asparagine, 2 grams of $NaNO_3$, and 45 grams of $KClO_3$ to 1 liter of basal medium. Initially, 1.6 grams of L-asparagine was added as an alternative nitrogen source, but in later experiments the alternative nitrogen source was changed from L-asparagine to 1.4 grams of L-threonine per liter. The change from L-asparagine to L-threonine increases the percentage of NitM mutants to be recovered (Klittich and Leslie, 1988). CZC was prepared by adding 39 grams of Czapek-Dox agar and 45 grams of $KClO_3$ to 1 liter distilled water. In this culture medium, the concentration of $KClO_3$ was increased to 6 % for isolates whose growth was not restricted to concentrations of 4.5 % $KClO_3$. To recover *nit* mutants, mycelial plugs (1-2 mm^3) from the margins of 4 to 6-day-old cultures grown on PDA were transferred to petri dishes containing MMC. Then, they were placed in the continuous dark condition at 26 °Ċ. After 7 to 21 days the *nit* mutants were recovered from the tip of the fast growing auxotrophic sectors. Approximately 40-60 chlorate-resistant sectors from each fungal isolates were selected on MMC and transferred to MM. Sectors which had sparse growth on MM (containing $NaNO_3$ as the sole nitrogen source) were considered mutants and were sub-cultured on selective media; hypoxanthine medium (HM), nitrite medium (NM) and ammonium medium (AM) for phenotypic classification into *nit1*, *nit3*, or NitM (Correll *et al*., 1987; Leslie and Summerell, 2006). Also, isolates with heavy growth on MM containing $NaNO_3$ as the sole nitrogen source were discarded. Such isolates are ither: the wild type parents that has escaped the screening; are isolates that are heterokaryons for more than one *nit* mutation; or *crn* mutants that can both utilize $NaNO_3$ and are resistant to ClO_3. To characterize *nit* mutants, five selective media were prepared. These media are as follows; nitrate medium (by adding $NaNO_3$ to the basal medium), NM (by adding $NaNO_2$ to the basal medium), HM (by adding $C_3H_4N_4O$ to the basal medium), AM (by adding ammonium tartarate $[(NH_4)_2C_4H_4O_6]$ to the basal medium) and uric acid medium (by adding $C_5H_4N_4O_3$ to the basal medium) (Correll *et al*., 1987). In the characterization of *nit* mutants, MM containing ammonium tartarate served as a positive control and the MM containing $NaNO_3$ served as a negative control (Leslie and Summerell, 2006). The plates were incubated at 26 °Ċ for 10 days. Then the mutants were phenotypically classified based on their mode of growth. The *nit* mutants could be divided into three phenotypic classes including; *nit1*, *nit3* and NitM (Table 2).

Table 2. Utilization of nitrogen sources for standard phenotyping of *nit* mutants after growth on MM + $KClO_3$ (Correl *et al*., 1987)

	Medium Supplement				
Strain Type	NH_4	NO_3	NO_2	Hypoxanthine	CLO_3
Wild type	+	+	+	+	-
nit1	+	-	+	+	+
nit3	+	-	-	+	+
NitM	+	-	+	-	+
Crn	+	+	+	+	+

Complementation test

Recovered *nit* mutant from fungal isolates were paired on MM containing $NaNO_3$ as a sole nitrogen source. Initially, each isolate was paired with itself and then with any other isolates. Test mutants were placed approximately 1 cm apart or more, if one of them was fast growing. Plates containing fungal isolates were incubated in the continuous dark condition at 27 °Ċ for 14 to 28 days. The plates were observed weekly for complementation evidence that is easily detectable by the formation of heterokaryons indicated by a dense aerial wild type mycelium where two mutants met (Gichuru *et al*., 2000). Isolates were considered to be vegetatively compatible if prototrophic growth was observed at the zone of anastomosis. Otherwise the isolates were determined as vegetatively incompatible.

Results

Nit mutant isolation

Nitrate non-utilizing mutants were identified as they show no heavy growth (without aerial mycelia) and having fast growing sectors on the tested chlorate containing culture media. All other isolates with no growth or having wild type growth (with dense and slow growing mycelia) on the tested chlorate containing culture media were excluded from the experiments. Of the forty-nine *V. sordida* isolates examined, 29 *nit* mutants isolates were recovered on MMC containing 4.5 % $KClO_3$ and 14 isolates on MMC containing 6 % $KClO_3$. For three isolates (G-91, 69-2 and G-105 red), *nit* mutants could not be obtained after enhancing the potassium chlorate up to 6 %. Three other isolates (335, G107 and 103) did not grow on MMC and therefore, they were excluded from the experiments. Also, none of the fungal isolates were able to grow on CZC and MMC amended with 50 mg/l rose Bengal even after 20 days keeping

them in continuous dark condition at 26 °Ċ (Table 3). Thus 43 isolates were selected for further experiments.

Table 3. Frequency and phenotype of nitrate non-utilizing (*nit*) mutants recovered in three culture media

Culture medium[a]		***nit* mutants classes**[c]		
	nit[b] (%)	*nit1*(%)	NitM(%)	*nit3*(%)
MMC	87.75	0	0	100
CZC	0	0	0	0
MMC amended with 50 mg/l rose Bengal	0	0	0	0

[a]MMC = minimal agar medium plus chlorate, CZC= Czapek-Dox agar plus chlorate, [b]Precentage of chlorate-resistant sectors that grew as thin expansive colonies on minimal medium, [c]*nit* mutant phenotypes determined according to growth on basal medium amended with different nitrogen sources.

Nit mutant phenotyping

Nit mutant phenotypes were identified after growth pattern of isolates on media that contained one of the five different nitrogen sources including: sodium nitrate, sodium nitrite, hypoxanthine, ammonium tartarate and uric acid. According to the results, all examined *nit* mutants on differential culture media containing different nitrogen sources had wild type growth on hypoxanthine and ammonium tartarate containing media and had poor growth on $NaNO_3$ and $NaNO_2$ containing media thus, all of these mutants were classified as *nit3*. No other types of *nit* mutants were detected according to their growth on differential culture media containing different nitrogen sources. The frequencies of NitM, *nit1* and *nit3* phenotypes were 0, 0 and 100 %, respectively. Mutations that affect the assembly of a molybdenum-containing cofactor necessary for nitrate reductase activity produce the NitM mutants and a mutation in nitrate reductase structural locus produce the *nit1* mutants. Forthermore, a mutation in nitrate assimilation pathway specific locus produce *nit3* mutants (Mohammadi and Mofrad 2010). Results showed that in the studied isolates, mutation in *nit3* locus affected the pathway specific regulatory protein and such mutants were not able to synthesize nitrate and nitrite reductase enzymes to utilize the NO_3 and NO_2.

Complementation test

Pairings are done on MM containing $NaNO_3$ with NO_3 as the sole nitrogen source. All *nit* mutants grew thinly on this medium unless they form heterokaryon with another compatible mutant. The prototrophic growth from

the pairings of different complementary auxotrophic mutants must be due to heterokaryosis. In all parings using *nit* mutants obtained from different fungal isolates, no prototrophic growth was observed after 14 to 28 days of incubation at 27 °Ċ indicating the presence of only one type of *nit* mutant (*nit3*).

Discussion

Cytospora canker is a serious fungal disease of many shrub, fruit and ornamental tree species in the urban areas, forests, orchards, and nurseries (Kepley and Jacobi, 2000). *V. sordida*, the causal agent of canker disease, is also one of the most important fungal species with wide host range and geographic distribution in Iran (Fotouhifar *et al*., 2010).

Nitrate non-utilizing mutants have been widely used to define VCGs and for studying genetic diversity in anamorphic/teleomorphic populations of many filamentous fungi (Leslie, 1993, 1996; Katan *et al*., 1994; Rowe, 1995; Correll and Gordon, 1999; Glass *et al*., 2000). Vegetative compatibility grouping are ideal markers for population studies, because they occur naturally, are easy to score using spontaneous *nit* mutants (Klittich and Leslie, 1988; Cai and Schneider, 2005). VCGs have been very useful for identifying clones in asexual fungi (Korolev and Gindin, 1999).

This is the first study to generate nitrate non-utilizing mutants for investigation of vegetative compatibility and heterokaryosis in *V. sordida* isolates. According to the results, all recovered mutants from 49 tested isolates on chlorate medium, were identified as *nit3* mutant based on their growth on differential culture media having only one nitrogen source. The results indicate that in all examined *V. sordida* isolates, the same mutation occurred in the nitrogen assimilation pathway. The lack of growth of *V. sordida* isolates on $NaNO_2$ and $NaNO_3$ can be attributed to a nonfunctional nitrate and nitrite reductase enzymes which are the essential enzymes in nitrogen assimilation pathway. In such mutants (*nit3*), mutation occurs in *nit3* locus which code for a pathway-specific regulatory protein affects the NO_3 utilization pathway. These mutants synthesize neither nitrate nor nitrite reductase, and can utilize neither NO_3 nor NO_2 as nitrogen sources. Instead, all tested *V. sordida* isolates were able to utilize hypoxanthine. However, the ability to use hypoxanthine rules out the possibility of a block in the molybdenum cofactor locus. Mutants of the pathway-specific regulatory gene for induction of nitrate reductase and nitrite reductase (*nit3*) are frequently isolated in *Aspergillus* as well as in *Fusarium moniliforme*, but infrequently isolated in *Neurospora* (Klittich and Leslie, 1988).

Pairings for complementation test were done on MM containing $NaNO_3$ as a sole nitrogen source. According to the results, no heterokaryons, as indicated by absent prototrophic growth, have developed between identified *nit*

mutants of the same isolate as well as between *nit* mutants of the different isolates. The isolates were incompatible with all other isolates and were not assigned with a VCG code indicating the isolation of only one type of *nit* mutant (*nit3*). So, it was not possible to group the tested fungal isolates in different VCGs. The results were predictable because common pairings for VCGs are done between *nit1* and NitM of the same isolates and between *nit1* and NitM of the different isolates to determine the self and non-self compatibility/incompatibility (Leslie and Summerell, 2006). Gichuru *et al*. (2000) studied the vegetative compatibility of 44 isolates of *Colletotrichum kahawae* that were obtained from different areas in Kenya. Phenotyping of recovered *nit* mutants on MMC and MEAC culture media revealed the dominant frequency of *nit3* mutants in comparison to other classes (*nit1* and NitM). Barcelos *et al*. (2011) investigated VCGs in 47 isolates of *Colletotrichum lindemuthianum* and generated 295 *nit* mutants on minimal medium supplemented with potassium chlorate and identified 279, 15 and 1, *nit3, nit1* and NitM mutants respectively. The 39 isolates produced only *nit3* mutants. It was mentioned that the high frequency of *nit3* mutant was also found in previous researches on *Colletotrichum* species done by Brooker *et al*. (1991) and Beynon *et al*. (1995). The recovery of *nit* phenotypes (especially NitM) from *Colletotrichum* species is clearly not straightforward and sometimes is not possible.

Jensen and Adams (1995) studied the nitrogen utilization pattern of two species of *Leucostoma* using virulent isolates of *L. persoonii* and *L. cincta* (now well known as *L. cinctum*) and two virus infected hypovirulent isolates of *L. persoonii* on 47 nitrogen sources. Virulent isolates of *L. persoonii* were able to utilize 38 nitrogen sources. *L. cincta* isolates were unable to use specially branched side chain aliphatic amino acids and the authors concluded that the ability to utilize such nitrogen sources might be a species specific characteristics. Virulent isolate of *L. cincta* could not utilize nitrate and nitrite. The two hypovirulent isolates of *L. persoonii* also had restricted usable nitrogen sources and were unable to grow on $NaNO_2$ and $NaNO_3$ and branched side chain aliphatic amino acids as the sole nitrogen sources. They concluded that inability to utilize specific nitrogen sources is likely the result of mycovirus infection. Mycovirus infection of Iranian isolates of *V. sordida* could be possible because, the recent investigation on *C. chrysosperma* isolates obtained from walnut trees in Iran done by Abbasi *et al*. (2012) revealed the presence of dsRNAs in some isolates.

The type of culture media affects the generation and the proportion of the generated different *nit* mutant classes. The standard is to use MMC with asparagine as the nitrogen source, but other culture media also can be used

(Leslie and Summerell, 2006). In this experiment, three types of culture media including MMC, MMC+rose Bengal and CZC were used to generate different *nit* mutants and alter their frequencies. Of the forty-nine *V. sordida* isolates, 43 isolates were able to generate *nit* mutants on MMC containing 4.5 % to 6 % $KClO_3$. None of the fungal isolates were able to grow on CZC and MMC+rose Bengal. Based on the results, MMC was detected as the most suitable culture medium for generation of *nit* mutants in the *V. sordida* isolates. Bayman and Cotty (1991) suggested a relationship between growth rate on chlorate-amended media and frequency of generation of *nit* mutants. Culture media that greatly restricted fungal growth yielded the most *nit* mutants. Adding rose bengal to the chlorate medium, increased *nit* mutant frequency in several fungi such as *Alternaria*, *Aspergillus* and *Fusarium* species. Apparently, the rose bengal increases the restrictive nature of the chlorate medium, permitting *nit* mutant sectors to be distinguished (Elias and Cotty, 1995). In this study, no *nit* mutants were generated from isolates grown on rose bengal-amended chlorate medium and this medium did not increase the frequency of *nit* mutants in *V. sordida* isolates. On rose bengal-amended chlorate medium, fungal colonies were greatly restricted to the space close to the primary transferred mycelial plugs without producing any fast growing sectors. Mycelia of *V. sordida* isolates on rose bengal-amended chlorate medium absorbed rose bengal and turned from hyaline to rose red. Adding rose bengal to the chlorate medium also did not increase frequency of *nit* mutant in some fungi. Bayman and Bennett (1998) mentioned inefficiency of adding rose bengal in chlorate medium increase the frequency of *nit* mutants in *Cunninghamella echinulata* var. *elegans*. Isolates of *Botryotinia fuckeliana* also did not produce sectors in rose bengal-amended chlorate medium and even though this medium restricted colony growth to the centre of the plates (Delcan and Melgarejo, 2002).

The *V. sordida* isolates as well as *Valsa* sp.1 and *Cytospora* sp.1 isolates that were morphologically very similar to *V. sordida* and *C. chrysoperma* respectively, were obtained from 15 different provinces with diverse topological and climatic traits and from almost 24 diverse host plants including shrubs, fruit and ornamental trees in Iran. Despite the considerable geographic and host diversity in studied fungal isolates, most of them generated the same *nit3* mutants on chlorate amended culture media. It was assumed that dominant asexual reproduction despite very rare sexual reproduction of this species in Iran and easy dissemination of the fungus primarily by plant materials in long distances even from one province to others resulted to gene flow in native populations of the fungus occupying a specific host plant or specific ecozones. In relation to nitrogen assimilation, gene flow resulted to homogenization of

different populations. As such, most of the fungal isolates obtained the same pattern in nitrogen utilization.

This study is the first investigation in *V. sordida* isolates from Iran on *nit* mutant generation, a tool that may be used for vegetative compatibility grouping. However, recovering of only one mutant type (*nit*3) did not allow for VCG testing. Screening more isolates of *V. sordida* on different chlorate amended culture media and even generating other kinds of mutants such as sulphate non-utilizing mutants (*sul*) on media amended with sodium selenate as done in *Fusarium oxysporum* f.sp. *melonis* (Jacobson and Gordon, 1998), *Magnaporthe grisea* (Harp and Correll, 1998) and *Botrytis cinerea* (Korolev *et al*., 2008) isolates, may result to better understanding of vegetative compatibility of *V. sordida* isolates.

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