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Isolation of Heterozygous Diploid Strain in *Alternaria Alternate* Fungus

Huda W. Hadi

Science College /Mosul University / Biology Department/

**Corresponding author:* hudaalhyali85@gmail.com

Abstract. In this study, 31 spontaneous or UV-induced mutants were isolated. Some of the mutants were white (*w*) (melanin missing), others were nitrate auxotrophic (*nit*); others were sulphate auxotrophic (*s*), and others combined the colored and auxotrophy genotypes. In addition, two strains one of them black Chl10 strain, nitrate auxotrophic, has the genotype (*nit10, w2 +, s2 +*) and the white Sel2 strains up hate auxotrophic, has the genotype (*nit10 +, w2s2*) both were used in genetic analyses in this fungus. The heterozygous diploid strain was isolated after heterokaryon formation in *Alternaria alternata* fungus. However, using heterokaryon and heterozygous diploid genetic analyses showed for the first time in this fungus that the basic steps for para sexual analysis are available in this fungus. This makes it feasible to do further genetic studies in this imperfect species to elucidate the genetic control of melanin and other secondary metabolites (e.g. host-specific toxins) which are thought to be essential for pathogenicity.

Keywords. Auxotrophic mutant, Color mutation, Chlorate, Selenate, Heterozygous diploid strain, Genotype.

INTRODUCTION

The genus *Alternaria* belongs to Imperfecti Fungi (Deuteromycotina) which either do not have sexual reproduction because it has not or is not discovered yet. Most of these fungi are opportunistic, especially in the post-harvest period. Through the production of mycotoxins, a group of diseases occurs in many economically important plants, such as the Gramineae family, crops, ornamental plants, and vegetables, which include potatoes, cauliflower, and carrots, or in fruits such as tomato, citrus, and apples (Agrios, 2005; Rotem, 1994; Thomma, 2003).

Because of many diseases caused by these fungi in many economically important plants and the role of specialized toxins, enzymes, and melanin in plant diseases, their species became a substance for many studies that explained their spread, classification, biology, and several genetic aspects related to their production of toxins, melanin and their relationship to its pathogenicity (Hadi, 2019; Simmons, 1992).

The present study aims to make a genetic study, dealing with the melanin production in Alternaria alternata fungus by the resistant mutants to chlorate and selenate. This is for building the heterokaryon, which is a main preliminary step for the study of the parasexual cycle in this fungus, and then isolating the diploid strain, which is the beginning of physiological and biochemical studies for the production of this dye and linking this to the pathogenicity or survival of this fungus.

The parasexual cycle is characterized as a method of producing new genetic structures (Recombinants) in fungi regardless of the usual sexual reproduction. Ponte Corvo and his friends in 1953 called this new method the Para sexuality in the *Aspergillus nidulans*, or the term Alternatives to sex (Haldane, 1955) to indicate this pattern in the fungi and other patterns that compensate for sexual reproduction in bacteria.

MATERIAL AND METHODS

Test Organism

AA1 Isolate from *A. alternate*, which is wild isolation in its nutritional needs and has black conidia were isolated from leaf spotted disease of Legumes plant in Mosul University/Science College/Biology Department. The rest of the spontaneous and induced mutants were derived or isolated from the parental strain AA1.

CulturalMedia

- Potato-Dextrose Agar medium (PDA): is used when the rapid growth of the fungus and the formation of a large number of conidia are desired (Pitt and Hocking, 2009).
- Potato-Sucrose Agar medium (PSA): The first medium itself was in terms of preparation and quantity except that dextrose was replaced by available sucrose.
- The Minimal Medium (M), was dependent on genetic studies that were used with *Aspergillus amstelodomi* and *Aspergillus nidulans* (Caten, 1979), keeping in mind that the amount of sucrose was 20 gm/L for *Alternaria alternata* (Hadi and Dhahi, 2012).
- Sodium deoxycholate (D): it was used to limit the growth of the fungus to give small, specific, and separate colonies when needed, when (D) salt is added to the media this media bear D in last of its abbreviated word like MD, PDAD, etc. (Caten, 1979; Mackintosh and Pritchard, 1963).

Isolation of Auxotrophic Mutants

The conidia of this fungus are multicellular; it is difficult to obtain auxotrophic mutants by culturing the conidia on a complete nutrient media such as PSAD and then transferring the growing colonies using a replica plating on a minimal medium (Lederberg and Lederberg, 1952). This requires that all single cells of conidia must be mutated from the wild type to the auxotrophic status because the mutant characteristic is mostly recessive (Auerbach, 1976).

For purely statistical matter, the possibility of multiple cell development in one direction is very unlikely, so there must be an automatic selection method (Automatic Selection) to completely isolate the mutant conidia from the wild to the auxotrophy pattern by killing the non-mutant conidia and keeping the mutant ones.

Potassium Chlorate ($KClO_3$) Resistant Mutants

Potassium Chlorate ($KClO_3$) Resistant Mutants were isolated by culturing in media containing killing and super killing concentrations of potassium chlorate after determining the minimal inhibitory concentration (MIC). Two types of resistant mutants were isolated spontaneously and induced by the UVC as the source of the radiation. We use a UVC wave lamp with a length of 253.7 nm type (Scottish Science NOP 189 m) as depended by (Hadi and Dhahi, 2012) based on. (Cove, a 1976; b 1976).

Selenate Resistant Mutants

Selenate Resistant Mutants were isolated in the same way as the chlorate-resistant mutants, except that sodium selenate was used and by inoculating the media containing killing and super killing concentrations of selenate, and according to the type of mutant if it was spontaneous or induced by ultraviolet radiation, as done in (Hadi and Dhahi, 2012) depending on (Arst, 1968).

Hetrokaryon Formation

The PDA medium was inoculated with the Chl10 and Sel2 strains by making two parallel lines in the same way as in (DeBertoldi and Caten, 1979). From the regions where the two strains converge, four cubes size 1-2 mm 3, are transported to the M that allows the heterokaryon to grow while the two strains do not grow in a medium that does not contain their nutritional needs (Carlile et al., 2001).

Isolation of the Heterozygous Diploids

After the growth of the heterokaryon on the M medium, the conidial suspension. This solution was used for inoculating the MD medium that the two strains of heterokaryon are not allowed to grow on it due to their nutritional needs. This medium allows the growth of strains of heterozygous diploid due to the phenomenon of complementation between the two nutritional needs of the mutations involved in their formation. Incubation of plates at 28 ° C for 3-4 days, after which diploid conidia were, distinguished (Roper, 1952).

RESULTS AND DISCUSSION

Auxorophic Mutants

After determining the treatment with UV rays that kill 90% of the treated conidia and minimal inhibitory concentrations of potassium chlorate that kill different strains of the fungus, several attempts were made to isolate chlorate resistant mutants from different strains (Hadi and Dhahi, 2012). The UV rays at the wavelength from 250-260 nm are a mutagen factor in all organisms (Drake, 1970; Auerbach, 1976). In this way, many chlorate mutants were isolated from the AA1 parental strain and several other strains derived from it (Hadi and Dhahi, 2012)

When the phenotype is chlorate resistance, so each of these mutants was given the initial symbol Chl and a small number next to it representing its serial number in isolation, its ability to grow

on the MM medium tested as well as on the MM medium-plus ammonium tartrate as a source of nitrogen. Thus, the (Chl5-Chl27) resistant mutants were isolated while the (Chl1-Chl4) mutants were neglected due to their poor growth in the chlorate media. Table 1 shows a summary of the characteristics of the mutants Chl27-Chl5.

Five UV-induced mutants could be also isolated from white conidia strain SW2. As these mutants were isolated based on their phenotype-selenate resistance, each of them was given the initial (Sel) symbol and added to each symbol representing the sequence of isolation such as Sel1-Sel5 mutants (Table 1).

It was purified by single spore isolation and its growth ability was tested on M medium, M + D-meth medium, and M + D-meth + Selenate medium (Table 1), which shows the totality of the phenotypes of parental strains and the different mutants that were isolated from the wild type AA1 or one of their derivatives and the methods of obtaining these strains.

Table 1. General characteristics of mutants isolated and studied.

Parental strain	Treatment type	Mutant strain	Conidia color	M+ tartrate + chlorate	M+Selenate + D-meth	M	M+Tartrate	M D-meth+
AA1			B	-	-	+	+	+
	UV	SW1	W	-	-	+	+	+
	UV	SW2	W	-	-	+	+	+
	UV	SW3	W	-	-	+	+	+
	0	Chl_5-Chl_9	B	+	-	-	+	-
	UV	Chl_{10}	B	+	-	-	+	-
Chl_{10}								
	UV	Chl_{11}	W	+	-	-	+	-
SW_1								
	UV	Chl_{12}	W	+	-	-	+	-
SW_3	0							
		Chl_{27}-Chl_{13}	W	+	-	-	+	-
SW_2								
	UV	Sel_5-Sel_1	W	-	+	-	-	+

0: Without treatment; UV: UV treatment; B: colony color is black; W: colony color is white; Chl: phenotype resistant to chlorate; Sel: phenotype is resistant to selenate; Chlorate: $KClO_3$ 100mM; Tartrate: Ammonium Tartrate (5mM); Selenate: 300mM Na_2SeO_4; D-meth: 0.2mM D-methionin; +:Growth; -: No growth.

Isolated mutants without treatment (0) were isolated after culturing the conidia of the parent strain and incubated on the isolation medium for approximately 15-20 days.

Whereas the aim of isolating the resistant mutants was not the resistance itself, but benefiting from its other side, which is the food deficiency (Auxotrophy) that each auxotrophic mutation represents, so this aspect was emphasized in both types of resistant mutations. In the case of potassium chlorate-resistant mutations (Chl), this appearance represents a nutritional need for

nitrogen except for nitrates (NO_3^-) (Cove, a1976; b 1976). Therefore, the growth of Chl mutations has been tested on the minimal medium M and the M medium supported by ammonium tartrate as an alternative nitrogen source for nitrates (NO_3^-). Mutations showed a negative response on the medium containing nitrates as the only source of nitrogen, while it showed positive growth when the medium was reinforced with ammonium tartrate in addition to nitrates, these mutations represented an auxotrophy of nitrogen.

Gene Nomenclature

As the mutation occurs in more than one gene that controls the pathway of taking nitrates from outside to the inside of the cell. Then, nitrates are metabolized to the ammonium inside the cell. It is leading to a nutritional deficiency of nitrogen (Cove, a1976; b 1976). So, all mutations were given Chl Genotype. The general initial symbol (nit) followed by a number representing the sequence of mutation isolation according to the gene nomenclature system for fungi and Aspergillus nidulans fungus that proposed by Clutter buck (1974), without knowing whether the nit genotype represents one gene or more (Table 1).

The same is said about the mutants, that are resistant to selenate (Sel), as they are mostly from the fungus A. nidulans and represent a nutritional deficiency of sulfur. Except for sulfates (SO_4^{-2}) and they were given the genetic symbol (s) which is found as several different genes through sulfur metabolism pathway starting from its entrance from outside to inside the cell, then sulfur-containing amino acids metabolism such as cysteine and methionine (Arst, 1968).

According to the nomenclature of this type of mutation in *A. nidulans*, the five mutants Sel1-Sel5 were given the (*s1-s5*) gene symbols (Table 2) without knowing whether these five mutations *s1- s5*represented one or several genes across the sulfate metabolism pathway SO_4^{-2}. However, this nomenclature is according to the genetic symbols that were used for the color mutations (the color of conidia) in the *A. nidulans* fungus (Clutter buck, 1974) and *A.amstelodami* fungus (Caten, 1979). Through, the genetic symbol (*w* +) was used to refer to the gene where its mutation to (*w)* in both species. It turns the green color of the conidia and the colony to the white color (W). It used the same symbol *w* + in the current study referring to the gene that gives melanin and the black color (B) in the *A.alternata* fungus, through which its mutation to *w*, stops the production of melanin and transforms the colony color (hyphae and conidia) from black (B) to white (W) (Table 2).

The study of this group of mutations (*w*) according to their genetic behavior represents an important aspect of the following research to find out the genes indicating the pathway of building melanin in this fungus.

Table 2. Phenotypes and initial genotypes of strains with their sources (Hadi and Dahi, 2012).

Phenotype of strain	Colony color	Genotype structure	number	Source of strain
AA_1	B	*w.t*	1	Wild type strain isolated from Legumes plant in Biology Dept./Sci. Coll./Mosul Univ.
SW_1	W	w_1	1	Gained by mutagenesis of AA_1 colonies by UV radiation
SW_2	W	w_2	1	Gained by mutagenesis of AA_1 colonies by UV radiation

SW_3	W	w_3	1	Gained by mutagenesis of AA_1 colonies by UV radiation
Chl_9-Chl_5	B	nit_9-nit_5	5	Spontaneous
Chl_{10}	B	nit_{10}	1	Gained by mutagenesis of AA_1 conidia by UV radiation
Chl_{11}	W	nit_{10} w_{11}	1	Gained by mutagenesis of Ch_{10} conidia by UV radiation
Chl_{12}	W	nit_{12} w_1	1	Gained by mutagenesis of SW_1 conidia by UV radiation
Chl_{27}-Ch_{13}	W	w_3nit_{27}-w_3nit_{13}	15	Spontaneous
Sel_5-Sel_1	W	w_2s_5-w_2s_1	5	Gained by mutagenesis of SW_2 conidia by UV radiation

B: The colony's color is black; W: the colony color is white; SW: a white color mutant; Chl: a mutant its phenotype is resistant to potassium chlorate; Sel: mutant with a sodium selenate-resistant phenotype; *w*: the genetic symbol for the mutation of white conidia; *nit*: the genetic symbol for the inability to benefit from nitrates (NO_3^-) as the only source of nitrogen in the growth medium (i.e., with an auxotrophy of nitrogen other than nitrate; *s*: the genetic symbol for the inability to benefit from sulfate (SO_4^{-2}) as the only source of sulfur in the growth medium (i.e., with an auxotrophy of sulfur other than sulfate).

Genetic Analyzes

In this study, we confirmed the basic steps of the parasexual cycle in this fungus. It is imperfect in which the sexual cycle did not become clear, therefore the genetic analyzes focused on the parasexual analyzes and that was described mainly for the fungus *A. nidulans*, and this parasexual cycle includes heterokaryosis and nuclear fusion between several nuclei to form the nucleus of the double chromosome group (Heterozygous diploid) (Sonneborn and King, 1974; Pontecorvo et al., 1953). Moreover, another aim is to establish a clear genetic way to study the genetic control of the melanin pathway production as it is one of the pathways of secondary metabolism that may play an important role in the fungus growth, maturation, and development conidial, survival, and pathogenicity (Rotem, 1994; Thomma, 2003). The strains detailed have been used for this purpose (Table 3).

Table 3. Properties of *A. alternata* strains and their sources that are used in Parasexual Analysis in the current research.

Strain	*Genotype structure*	*Conidia color*	*Growth on M medium*	*Source of strain*
AA_1*	*w.t.*	B	+	*Wild isolation of Legumes leaves*
SW_2	w_2	W	+	*A white strip from irradiated AA_1colony*
Chl_{10}	nit_{10}	B	-	*Resistant Isolate to potassium chlorate from irradiated AA_1 conidia*
Chl_{11}	nit_{10},w_{11}	W	-	*White isolation of irradiated Chl_{10} conidia*

Sel_2	s_2 w_2	W	-	*Resistant Isolate to sodium selenate of irradiated SW_2 conidia*

w: a mutation that converts the conidia color from black (B) to white (W); *nit*: a mutation that makes the individual resistant to potassium chlorate (Chl) and at the same time creates a nutritional deficiency of a nitrogenous source other than nitrate (NO_3^-); *s*: A mutation that makes an individual resistant to sodium selenate (Sel) and at the same time creates a nutritional deficiency of a sulfur source other than inorganic sulfate (SO_4^{-2}); *All remaining strains were induced as shown in the table. The conditions of irradiation with UV radiation were as described in (Hadi and Dhahi, 2012).

Heterokaryon Formation

Heterokaryon has been formed among many mutant strains but did not succeed except between Chl10 and Sel2 strains. Perhaps this is due to the unavailability of the vegetative compatibility characteristic (Moore and Frazer, 2002) in strains that are unable to form heterokaryon in contrast to the two strains that succeeded in forming the heterokaryon (Fig. 3).

During a standard period of four days, these two strains carried two types of genetic markers: the first of them were color genetic markers as the Chl10 strain had black conidia (Fig.1), while the Sel2 strain is white of conidia (Fig. 2).

The second type of marker is the Forcing biochemical marker. it means that the two strains carry different auxotrophic mutations that complete each other in their nutritional needs in maintaining continuity of heterokaryon on the minimal medium (M) and preventing its breakage into its components.

In this case, it is called Balanced heterokaryon (Pontecorvo et al., 1953; Webster and Weber, 2007). This is what had happened with the heterokaryon, which is formed between the two strains Chl10 and Sel2 when transferring it to the (M) medium. This allows just the heterokaryon to grow while Preventing the growth of the two parental strains because they do not contain their nutritional needs (ammonium tartrate as a source of nitrates and methionine acid as a source of sulfur).

However, the process of heterokaryon formation in this fungus is not easy. Therefore, only Tsuge and his group performed it in 1987 using the NTG carcinogen. Then, the researchers resort to analyzing the cellular walls of the *Alternaria alternata* and then forming the heterokaryon with a process called (Protoplast fusion), which depends on Chemical processes that don't have a relationship with isolating mutants by traditional methods (Prub et al., 2014; Saha et al., 2012; Wenderoth et al., 2017). Thus, the process of heterokaryon formation in this phytopathogenic fungus is the first step in parasexual genetic analysis. As well as a fast and effective method for conducting Dominance and Complementation tests (Fincham et al., 1979).

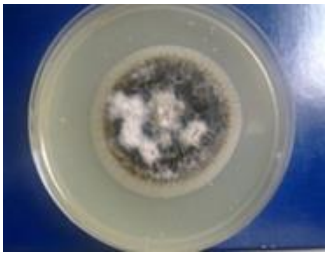


Fig.1. Chl_{10} strain (nit_{10}, $w_2^+s_2^+$) black conidia with an auxotrophy of nitrogen (*nit*).

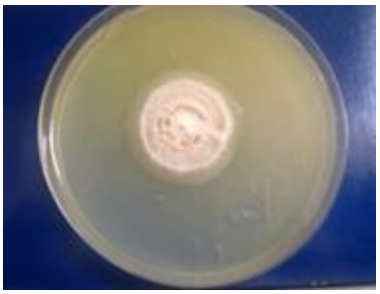


Fig.2. Sel_2 strain (*nit*$_{10}$+, *w*$_2$ *s*$_2$) strain of white conidia with auxotrophy sulfur (*s*$_2$).

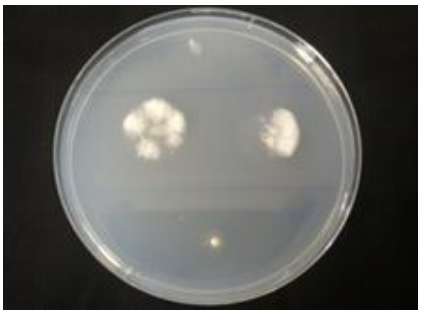
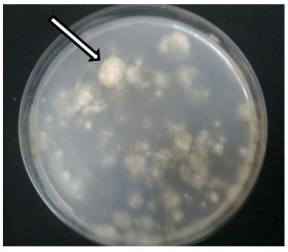


Fig. 3. Heterokaryon between the two strains Chl_{10} and Sel_2 (shown in Fig. 1) after four-day growth on M medium.

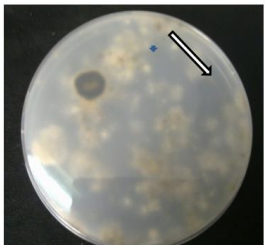
Isolation of the Heterozygous Diploid

Roper (1952), has demonstrated that a nuclear fusion can occur at low frequencies (10-6) between the haploid nuclei of the heterokaryonin *A. nidulans*. Thereby creating a heterozygous diploid nucleus that can be isolated based on its ability to grow on the minimal medium on which neither the haploid parental strains grow on, as well as using complementary color markers so that the diploid color colony can be distinguished from the colors of both haploid parental strains. Such a nucleus (strain) a double nucleus (Diploid) was considered an essential step in conducting parasexual genetic analyzes in *A. nidulans* (Sonneborn and King, 1974; Pontecorvo et al., 1953) and *A. amstelodami* (DeBertoldi and Caten, 1979; Dhahi, 1978).

It was confirmed that the white allele *w2* in the Sel2 strain had been set recessive in front of its wild allele *w2+* in the Chl10 strain by isolating the heterozygous diploid from the heterokaryon (Sel2 + Chl10). Through shoveling its conidia in sterile distilled water, preparing a thick suspension from them, filtering through sterile cotton wool, and then inoculating it on plates of MD media. On the dishes, white weak growths were formed, also other weak black and one black large colony (Fig.4).



a) The top of dish



b) The bottom of the dish

Fig. 3. Colony of a heterozygous diploid strain (makers by arrow A and B) from heterokaryon (Chl_{10}+ Sel_2).

This was interpreted by the fact that the weak white growths represent the white parental strain Sel2 (*w2, s2*) and the weak black growths represent the growing colonies of the black parental strains of Chl10 in the heterokaryon. After, their isolation during conidia formation in heterokaryon Because of each conidia needs a sulfur source (*s2*) except sulfates or a source of nitrogen (*nit10*) except the nitrates available in the MD medium. Consequently, they gave just a weak growth on this medium, and, when one of them was white (Sel2), the weak colonies, were white or black (Chl10). This means that the heterokaryon might be broken during the formation of conidia into its components Sel2 and Chl10

As for the large black colony, it was considered as the heterozygous diploid strain that resulted from the nuclear fusion between the two haploid nuclei parental strains of the heterokaryon. This heterozygous diploid strain was able to grow on the MD media because of the complementation between the two auxotrophic mutations *s2* and *nit10* and it gave the black color. Because of the dominance of the *w2+* allele in was strain Chl10 over the *w2* mutation in the Sel2 strain, so melanin pathway is completed and this dye is precipitated in the hyphae and conidia of heterozygous diploid strain.

CONCLUSION

The results of this current research showed that the heterokaryon formation, heterozygous diploid strain in *A.alternata*, all are essential requirements for conducting parasexual analyzes, that all are available in this fungus and they are a source of the confusing genetic variations in this fungus.

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