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Review Article

SECONDARY METABOLITES IMPORTANCE IN *ALTERNARIA ALTERNATA* FUNGUS

Huda W. Hadi

University of Mosul/College of Science/Biology department/Iraq. Email: hudsbio114@uomosul.edu.iq, hudaalhyali85@gmail.com

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ABSTRACT

The *Alternaria alternata* is from high metabolically active fungi, thus its metabolic products are closely associated phytopathogenicity. Consequently, this phytopathogen causes economic losses in crops, vegetables and fruits. The plant diseases are associated with human and animal health because metabolic products are metabolized within the bodies of living organisms. Melanin has a great role in the fungal resistance to various harsh environmental conditions. Starting from these points, the following article tries to shed some light on some details about the biology, pathogenicity, morphological characteristics of this fungus, and important secreted metabolites towards its hosts.

Keywords: *Alternaria alternata*; melanin; secondary metabolism; toxins; conidia.

Abbreviations:
Host Specific Toxins: HSTs; Non- Host Specific Toxins: Non-. HST; Tri Carboxylic Acid: TCA
Polyketides: PKSs; Alternariol monomethyl ether: AME; Alternariol: AOH; Tenuazonic Acid: TeA
Tentoxin: TEN; High Performance liquid Chromatography-diode detector: HPIC-DAD
Tetrahydroxynaphthalene: THN; dihydroxynaphthalene: DHN; Ultra Performance Liquid Chromatography: UPLC
Chromatography- Tandom Mass Spectrometer: MS / MS; Polymerase Chain Reaction: PCR
3,4 Dihydroxyphenylalanin : DOPA; y-glutaminyl-4-dihydroxybenzene named: GDHB
Wild type: w.t; ultraviolet radiation type B: UVB

1. INTRODUCTION

Alternaria alternata has two important features one of them is the production of melanin and bio-synthesis of many HSTs or Non-. HSTs, these secondary metabolites play a role in the pathogenicity of hosts (Barkai Golan, 2008; Kimuraand Tsuge, 1993). The genus *Alternata* contains plenty of species (Simmons, 1992), which can be found growing in different environments and from different parts of the world. It belongs to the class of Hyphomycetes fungi from sub-division Deutromy- cotina of the Eumycota division (Ainsworth *et al*., 1973). This genus is saprophytic fungi that play an important role in the decomposition processes of organic matters in nature, due to its derivatives of cellulose enzymes and other analytic enzymes. However, a significant proportion of them is opportunistic pathogens. Many diseases occur in economically important plants like legumes, vegetables and fruits (Agrios, 2005, Thomma, 2003). Consequently, the elimination process or control of this fungus is very difficult. There fore, the current article aims to shed light on this phyto-pathogenic fungus in terms of its biology, morphology, pathogenicity and then it's important metabolic products with some biochemical pathways and raw materials that begin with these important pathways in this fungus.

1.1 *Alternaria* Pathogenicity: It hasn't the sexual stage, but the fungus can resist as spores or mycelium on the remains of dead plants for a reason- able time or they cause latent infection within the seeds (Saeed and Juber, 2016). If the seeds germinate, the fungus attacks the plant. In another case, the seed growth makes fungus spread by air to other plants surfaces then infection occurs. The infection can also be hidden and are almost as invisible as when pomegranate infection (Ezra *et al*., 2019). Qualitatively, wounded vegetative tissues, weak, or stress exposed are more susceptible to *Alternaria* diseases than in healthy tissues (Thomma, 2003). At the beginning of the plant infection, the fungus infects the leaf veins in the form of rosy red spots deep in the middle with the development of spot disease changes colour to the brown. Patches can appear anywhere, whether leaf, bud or flower (Chelkowski and Visconti, 1992).

Infected buds are dry, then become dark compared with non-infected buds (Yousefi and Shahri, 2009), as for tree trunks injury, it leads to the internal tissue destruction, which is composed of tree trunks. It also affects the leaves and leads to yellowing and fallout, when the tissue sections of those injuries have been doing (Abd-Allah and Salih, 2008).

Alternaria species are the most important post-harvest pathogens they can infect fruits and vegetables, during the infection period many of them are capable of producing many fungal toxins that affect humans and animals (Apangu *et al*., 2018, Barkai-Golan, 2008, Stocco *et al*., 2019).

1.2. Secondary Metabolism: Is a general term covering a wide extent of metabolic processes that lead to production of metabolites which seem seemingly for durability or organism's survival that could be lost without any apparent effect on his life (Fox and Howlett, 2008). These pathways are effective when slowing down or stop the organism's growth. Precursors of pathways are middle compounds from the primary metabolic pathway, which build important molecules for life as proteins, nucleic acids, and amino acids. Other simple compounds (Intermediates) are not used by another primary metabolism, but it converted to other lines. For this reason, secondary metabolism often

called the fonts conversion pathway (Shunt metabolism) (Deacon, 1980).

Secondary metabolism is the structural process that needs energy leads to the production of specialized compounds, which is not necessary for the growth and development of the organism and it has specialized with certain species and strains. These compounds like citric acid that is the primary metabolite derived from primary metabolic intermediates with special pathways are produced excessively in the fungi (Dube, 1983).

There are three phases of growth in fungi: phase equilibrium (balanced phase) through it fungi growth increases and moderately nutrients proportions are taken. Running out of nutrients entering the storage phase collects fat and carbohydrate. The evidence on this point increased dry mass, constant mass and metabolites production until the medium depletion, these are markers of organism entrance in the stage of maintenance (maintenance phase) which considers secondary metabolites producing phase. Several studies regarded balance and storage one phase (trophophase), fungus grows without secondary metabolites formation through this phase and ends with the finish of basic nutrients such as nitrogen and phosphorus from the medium, then fungus begins to enter into stagnation or inactivity (Idiophase) within it secondary metabolites produces even disintegration incipience (Dube, 1983). Therefore, some researchers called secondary metabolites as (Idiolites) according to the Idiophase (Demain, 1986).

Nutrient termination runs the various biochemical pathways and this change in primary metabolism to the secondary metabolism prevents the poisoning from the intermediate chemical compounds of the primary metabolites collected in its cells, secondary metabolites generation can contribute in differentiation stages and sporogenesis or conidiogenesis in fungi. Most secondary metabolites are commercially useful materials such as antibiotics, but a number of them are harmful to humans and animals such as mycotoxins (Isaac, 1997).

Despite the high diversity in the chemical structure of secondary metabolites, they are divided into groups, depending on the origin of the biosynthesis. This reflects the small number of groups in which low molecular weight compounds are classified, as the raw material components of the cell, including nucleotides, amino acids, sugars, Acetyl-CoA and related compounds involving the TCA cycle intermediates like tricarboxylic acids and Terpenes. Microorganisms have a number of amino acids during growth pumped into the protein biosynthesis pathways as in the cell components building, such as nucleotides, heams, and sterols. Amino acids biosynthesis cannot stop when the cell growth slowdown therefore, turning these intermediates to produce secondary metabolites including Penicillin, Cephalosporin, Peptide antibiotics, and toxins (Rose, 1979). Notably, few secondary metabolites are made up from the ribonucleotides and deoxy-ribonucleotides collected by the organism during the growth period (Bilgrami and Verma, 1981, Craney *et al*., 2013, Rose, 1979). Moreover, secondary metabolites are deriving from one of three types: first PKS, the second is a combination of PKS and non-ribosomal peptides. The third is deriving from different pathways from the previous two (Brakhage, 2013).

PKSs pathway occurs in asexual spores (conidia) of *Aspergillus nidulans* leads to production of structurally unknown pigments (Ylihonko *et al*., 1996), whereas in *Apsergillus parasiticus* PKSs contributes to producing intermediate compounds for Aflatoxins biosynthesis (Lombo´*et al*., 1996). Pentaketide is a basic material of the 1, 3, 6, 8-tetrahydroxynaphtalene a resource of fungal melanin biosynthesis in *Alternaria alternata* fungus (Kimura and Tsuge, 1993). The encoded output by PKS synthetases genes plays a role in the secondary tracks. Used PKS steps containing (Multifunctional enzymes) that have programmed work. This term defines a clear control of the variables that determine the composition of the PKS special product. These variables are the selection of the starting unit in terms of unit type and number in the extended chain, as well as control the reduction cycle on the keto group for developing a carbon chain, which in turn establish the active chemical groups in each carbon chain (Hopwood, 1997). Genetic studies have a significant role in the study of secondary metabolic pathways; therefore, studies are seeking to access genetic information either selectively or otherwise to promote the production of secondary metabolic materials of medical importance (Craney *et al*., 2013). Mainly the secondary metabolism enzymes privacy is not absolute, it is relative so that one intermediate can be converted by two different enzymes leading to what is known as the metabolic grid net-work (Dube, 1983).

Responsible genes of secondary metabolism in fungi gather to form clusters and include two genes or more generally contributing to the metabolic pathway stroll through encoding for producing enzymes, transcription factors, and vectors (Keller and Hohn, 1997; Woo *et al*., 2010). *A. alternata* has genes that encode for toxin building such as ACT toxins that are present within the cluster in a single small chromosome called with (dispensable Chromosome). This Chromosome is unnecessary for growth but becomes essential for growth under certain specific circumstances whereas it is responsible for disease and the rot occurrence in young plant leaves and fruits (Stuart *et al*., 2009).

The secondary metabolic pathway spread via PKS of *A. alternata*; this pathway activity is not limited track on somatic hyphae cells but also works in conidia. In the cells of the fungal mycelium, this pathway produces specific toxin such as AOH and AME which produces in the newly developing conidia and did not know whether such a unique feature found in *Altrnaria* or other fungi (Häggblom, 1987; Hiltunen and So "derha" Ll, 1992). In *A. alternata* there are seven patterns (Pathotypes) each pathotype results dedicated toxin concerned to its host HSTs (Ito *et al*., 2004).

Turning to black pigment melanin is produced by *A.alternata* from acetate via PKS pathway 1, 3, 6, 8 THN as an intermediate compound to 1.8 DHN which after two reduction cycles and dehydration DHN generate and polymerize to melanin which has special functions (Bell and Wheeler, 1986, Kimura and Tsuge,

1993). On this basis, we will get to the toxins and the melanin as secondary metabolism compounds in this fungus. Secondary metabolism not only contribute to the pathogenicity such as producing mycotoxins or environmental protection and competition with other species but also in the development stages of the fungi. The majority of secondary metabolites are produced after completion of fungus growth, then sporogenesis stage starts is essential to the vitality of spores (Calvo *et al*., 2002). Furthermore, regulator proteins from secondary metabolites belonging to (Velvet) family, that play a role in coordinating and differentiation of sexual processes and sporogenesis , as well as control the production of secondary metabolism itself in *Aspergillus nidulans* (Bayram *et al*., 2008).

Secondary metabolism has other benefits as Andersen and Thrane in 1996 have been isolated *A. alternata* group from *A. infectoria* through production different metabolites from each, metabolites produced in *A.alternata* are alternariol, AME, AOH, altertoxin with two types I and II, and TeA. *A. infectoria* group produces a range of undiagnosed metabolites with several common metabolites between the two groups, identified metabolites by HPIC-DAD (Andersen and Thrane 1996).

1.2.1 *Alternaria alternata* Toxins*:* Mycotoxins are organic compounds produces from secondary metabolism, they are nonessential to the fungal growth process directly. Almost all the secondary metabolites of *A. alternata* species are mycotoxins through which the host defences penetrate, then kill the target cells and obtain the necessary nutrition for growth and vital processes (Howlett, 2006). There are several pathotypes of *A. alternata* that attack different plants; each of them produces specific mycotoxins for the specific hosts. Examples of these toxins and their hosts, is an AK toxin which causing black spot on Japanese pear fruit, AAL toxin produced by Pathotype causes tomato trunk warts, AF toxin infect strawberry, AM toxin arise from a pathotype that infects Apple, ACT toxin produced by the Tangerine pathogen, ACL toxin produced by the rough lemon pathogen, finally HSTs which intrudes on cane sugar (Agrios, 2005). The theory of *A. alternata* pathotypes was strengthened by molecular analysis. Producing toxins are chemically diverse, ranging from secondary metabolites with low molecular weight to the peptides (Kusaba and Tsuge, 1997). *A. alternata* produce all or part of the toxins listed in host cells, but with varying concentrations, one of them is higher than other concentrations. Thus, these toxins sequenced by different concentrations within the host (Guo *et al*., 2019, Noser *et al*., 2011, Patriarca *et al*., 2007, Scott *et al*., 2012). Moreover, a single isolate of *A. alternata* can infect two different hosts by producing two different HSTs (Masunaka *et al*., 2005).

The toxins working on different goals or one within the single cell, these toxins will eventually lead to the death of the plants. The ACT, AF and ACTG toxins infect the plasma membrane and cause loss of permeability property. While, AM toxin affects not only the plasma membrane but also the chloroplast through the destructive effect of thylakoid inhibitory electron chain and oxygen transfer rates with the final result to photosynthesis inhibition (Dai *et al*., 2004; Kohmoto *et al*., 1984; Otani *et al*., 1995). ACT and AT toxins affect mitochondria (Otani *et al*., 1995), whereas ACR induces mitochondria bulging and makes other morphological modifications on it and increases the oxidation of NADH, which follows by the loss of the Plasma membrane regularity which leading to electrolytes loss then necrosis (Akimitsu *et al*., 1989).

Depending on the specificity, toxins produced by fungi may be the specific to a particular host or not. None specific toxins work on supporting the pathogenicity only like virulence factors and enzymatic processes of causative agents (Ballio, 1991).

Chromatographic techniques are the leading techniques in the diagnosis of fungal toxins in general and *Alternaria* toxins in particular like UPLC (Noser *et al*., 2011), HPLC-DAD (Anderson and Thrane, 1995) and the MS/MS (Noser *et al*., 2011, Scott *et al*., 2012, Guo *et al*., 2019). Some of these toxins concentrations have been also detected using PCR in hepatocytes with a concentration of 50 mM (Hessel-Pras *et al*., 2019).

Many of *Altrnaria* non-specific toxin has been studying in some detail like tenuazonic acid, TEN and zinniol those are examples of producing toxins by several species of *Altrnaria*. However, there are several models were proposed for the explanation toxic effect of these toxins. Some examples Brefeladin a causes rupture of the Golgi apparatus, and inhibit the excretion. Whereas Curvularin is a cell division inhibitor by glycolysis of microtubules as a result of protein bio-synthesis inhibition as well as, Tenuazonic acid, which also inhibits protein biosynthesis, while zinniol affect membrane permeability (Fujiwara *et al*., 1988, Meronuck *et al*., 1972, Robenson and Strobel, 1981, Thuleau *et al*., 1988).

Genetic mutations play an important role in identifying the role of toxins in pathogens or virulence because these mutations lead to the dissection of biosynthesis pathways by collecting and knowing the information about the sequencing of the genes responsible for coding and building these toxins (Howlett, 2006; Keller *et al*., 2005).

1.2.2. The Melanin: Melanin is dark pigments; black colour that are formed in living organisms of all ranks from microbiology to higher organisms including humans. Melanin is not necessary for growth or evolution, but it promotes viable, competitive capabilities of the species and it provides organisms with protection from environmental stresses. It also may increase the virulence of pathogenic organisms that carrying it (Jacobson, 2000).

Melanin -producing genes in many cases gat-her into batteries (operons). Biosynthesis of melanin has been studying extensively in animals because of its association with skin diseases. DOPA Melanin is formed in animal cells by oxidation of tyrosine amino acid (Bell and Wheeler, 1986) this pathway occurs in fungi as well (Nurudeen and Ahearn, 1979). When DOPA reacts with amino acid cysteine, a DOPA cysteinyl compound composed to form red colour pigment, due to the similarity

of chemical origin for both dyes referred to them Phaeo melanin. Animal melanin produces in specialized cells by special organelles called melanosomes or melanin granules (Bell and Wheeler, 1986).

On the contrary, the melanin in fungi is pre-sent in the cellular walls, whether in hyphae or spores and then it is called melanin linked to the wall (Wall-bound melanin). The fungal melanin may found outside the cell wall in fibres or granules compose of the secretion of phenols outside to oxidize externally by released enzymes from fungus later (Bell and Wheeler, 1986; Nosanchuk *et al*., 2015). *A. alternata* is known to produce melanin and compiles it in cellular walls of the fungal mycelium, as for its location within the cell wall of conidia; it is confirmed by the use of electron microscope studies and other studies (Camp-bell, 1969; Carzaniga *et al*., 2002; Kheder *et al*., 2012). In other fungi, melanin exists only in fungal mycelium and the spores are free of it, such as the *Magnaporthe grisea (*Kawamura *et al*., 1997).

Melanin is spread in many fungi such as *Streptomycesscabies, Rhizobiumphaseoli* and ascomycetes fungi. Isolation and purification of melanin that result, it contains units of carbohydrates, protein, and fat. Melanin core contains hydroquinone and quinone. Relying on melanin composition, DOPA melanin can be produced from fungi as in mushroom (Bell and Wheeler, 1986) this type of melanin is also found in *Aspergillus* nidulans (Bull, 1970), or GDHB melanin produced by Basidiomycota fungi like *Agaricus bisporus*. Shikimic acid is the precursor for this melanin (Rast *et al*., 1981). The other type of fungal melanin is catechol melanin produced in the *Ustilago maydis* as the catechol is the basic unit in the construction of this melanin (Bell and Wheeler, 1986; Piattelli *et al*., 1965).

DHN melanin is produced by polyketide pathway referred also as a pentaketide melanin pathway that begins to collect five ketide units by a polyketide synthase enzyme these units build from five of acetate molecules. Recycling of the resulting compound by previous enzyme itself formation 1,3,6,8 THN compound and by reducing the component structure and dehydration turns into 1, 3, 8 THN. In the same way, the reduction in volume and dehydration turns the latter into 1.8 DHN, the main component of melanin (Plonka and Grabacka, 2006).

Melanin, caused by PKS through the DHN pathway, is the most common type of fungal melanin, but it is characteristic of fungal melanin and *Alternaria* melanin (Bell and Wheeler, 1986 Woloshuk *et al*., 1981).

DHN pathway detected by mutations that are induced or by using melanin biosynthesis inhibitors. Studies suggest a similarity of products and enzymatic activities of the DHN pathway in various well-described melanin fungi. Gene sequencing and genetic complement studies provide significant support for this pathway in several fungi such as *Magnaporthe grisea, Cochliobolus heterostrophus, and C. Lagenarium* and *Aspergillus* spp., among them, is *Alternaria alternata* (Bell and Wheeler, 1986). Mutations in genes that control melanin production are a powerful and effective tool for dissecting melanin production (Plonka and Grabacka, 2006). Via melanin mutants in *Alternaria alternata*, the melanin production pathway had explained in this fungus, starting with acetate, through several intermediate pathways, determining by the formation of DHN and controlled by three main genes, BRM1, BRM2, and ALM (Kimura and Tsuge, 1993) Fig.1.

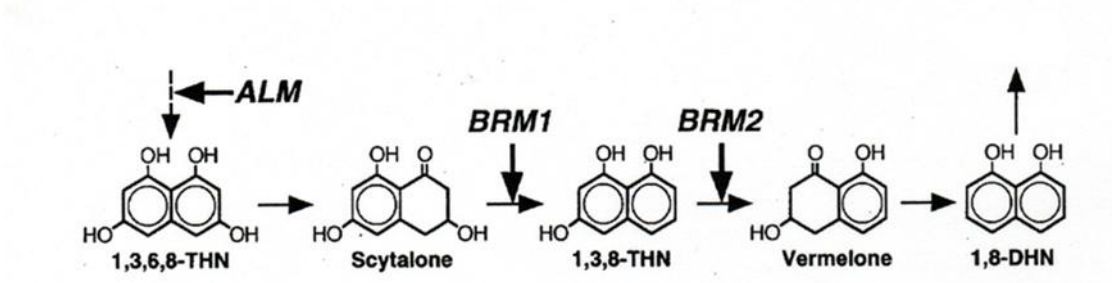

Figure 1: pathway of melanin production in *Alternaria alternata* and other fungi. Three genes control the steps of this pathway ALM, BRM1, and BRM2 (Kawamura *et al*., 1999).

Melanin mutants in *Alternaria* fungus differ in the colony colour from the w.t, as they are pure white. other mutants strains, weak pink colour, so is these strains are golden light brown colonies on the potato extract agar medium, in contrast to w.t which are black (Campbell *et al*., 1968, Tanabe *et al*., 1995).

Many functions have been assigning to melanin in the organisms that carry it (Bell and Wheeler, 1986; Jacobson, 2000). In plant pathogenic fungi, most of them have specialized structures called Appressoria, which helps penetrate the cell wall of plant cells. It is believed that melanin helps these appressoria to do their work as in the *M.grisea* and other pathogenic fungi and many consider it a virulence factor (Jacobson, 2000; Kheder *et al*., 2012). In the *A. alternata*, it has believed that melanin has not been relating to pathogenicity because the appressoria do not contain melanin. Also by comparing the pathogenic lesions on plant leaves such as the size and number of spots produced by the w.t and the mutant strains of melanin for fungus as they are at the same level (Kawamura *et al*., 1997; Kimura and Tsuge, 1993; Tanabe *et al*., 1995). Because of these points, many studies target melanin as the main factor in the fungal pathogenicity through several methods, including the use of biological control methods such as control by bacteria or fungi that have the ability to stop the melanin production or the worms and small animals using for feeding on those melanized fungi (Butler *et al*., 2005).

However, this does not negate the large role of melanin in the development and growth of conidia because melanized conidia of mutants are smaller and have fewer longitudinal and transverse septae than w.t conidia (Kawamura *et al*., 1999). This repeat in the *Mycosphaerella graminicola*, melanin mutants showed morphological changes, such as swelling of the mycelium, and they became less productive for aero mycelium on agar dishes (Choi and Goodwin, 2011). Melanin naturally appears to play a direct or indirect role in pathological processsses. It is important to study the mechanism of melanin construction or mechanisms of stopping it by knowing the genes that control the production and stop work (Butler *et al*., 2005a).

Melanin is an antioxidant factor due to a large number of methyl groups within its structure, which inhibits the peroxidase enzyme oxidative intermediates (Shcherba *et al*., 2000). When *A. alternata* has treated with oxidizing permanganate, hypochlorite or hydrogen peroxide, the melanized w.t were able to equalize these oxidants while albino the albino mutants were unable to do so (Jacobson *et al*., 1995). The melanin in *Aspergillus fumigatus* conidia plays an important role as a virulence factor protects the fungus from the host's immune response because it aggregates the cell walls of the conidia (Pihet *et al*, 2009). The presence of melanin in human pathogenic fungi like *Paracoccidiodes brasiliensis* reduces its sen-sitivity to host defences as well as fungal antibiotics (Taborda *et al*, 2008). This dye also protects against non-ionizing radiations such as ultraviolet radiation, when the melanocytic fungi exposed to lethal and under lethal dose of these rays. It is believed that the chemical composition of melanin cause electronic complexity, that allows it to dissipate photons and electrons released from radiation (Dadachova *et al*., 2007, Kheder *et al*., 2012, Luo *et al*., 2018). Therefore, the increase in absorption values was observed by melanin when UVB doses were increased when the *Alternaria solani* was irradiated (Fourtouni *et al*., 1998) Some also attribute the resistance of the crystals to ultraviolet radiation not only for the presence of melanin but also for the large size of the Alternaria spores (Kumar and Anal, 2018).

Melanin is also able to bind to various chemical compounds such as fungal antibiotics such as itraconazole and voriconazole. The chemical composition of melanin has not affected when incubiting with these antibiotics (Nosanchuk and Casa-devall, 2006). Numerous melanin properties are used to detect its location in fungal cells, such as its copper bonding properties. The fungal melanin is capable of copper bonding to form copper sulp-hide, which appears as a bright black dye that can be enhanced with silver. This method shows melanin deposition patterns in the fungal mycelium and is suitable for electron microscopy and photovoltaic screening, while white albinos or strains treated with melanin inhibitors such as Tricyclazole do not stained by this way (Butler *et al*., 2005b, CaesarTonthat *et al*., 1995).

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