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Toxin-based in-vitro selection and its potential application to date palm for resistance to the bayoud *Fusarium* wilt

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Abstract

Date palm (*Phoenix dactylifera* L.) is qualified as a 'tree' of great ecological and socio-economical importance in desert oases. Unfortunately, it is being decimated, especially in Morocco and Algeria, by a fusariosis wilt called bayoud and caused by *Fusarium oxysporum* f. sp. *albedinis* (Fao). Controlling this disease requires the implementation of an integrated management program. Breeding for resistance is one of the most promising component strategies of this program. Few naturally resistant cultivars with a mediocre fruit quality (dates) are known. Conventional and non-conventional methods are under development and have to use the simplest and easiest methods to screen for resistant individuals. The use of pathogen toxins as selective agents at the tissue culture step might be a source of variability that can lead to the selection of individuals with suitable levels of resistance to the toxin and/or to the pathogen among the genetic material available. Foa produces toxins such as fusaric, succinic, 3-phenyl lactic acids and their derivatives, marasmins and peptidic toxins. These toxins can be used bulked or separately as selective agents. The aim of this contribution was to give a brief overview on toxins and their use as a mean to select resistant lines and to initiate a discussion about the potential use of this approach for the date palm–Foa pathosystem. This review does not pretend to be comprehensive or exhaustive and was prepared mainly to highlight the potential use of Foa toxins for selecting date palm individuals with a suitable resistance level to bayoud using toxin-based selective media. ***To cite this article: A. El Hadrami et al., C. R. Biologies 328 (2005).***

Résumé

Le palmier dattier (*Phoenix dactylifera* L.) est un « arbre » d'une grande importance écologique et socio-économique dans les oasis des régions désertiques. Malheureusement, cette plante est décimée, essentiellement au Maroc et en Algérie, par

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une maladie vasculaire nommée bayoud et causée par *Fusarium oxysporum* f. sp. *albedinis* (Fao). Pour contrer cette maladie, l'implémentation d'un programme de lutte intégrée est recuise. La composante d'amélioration génétique de ce programme paraît être la plus prometteuse. Peu de cultivars présentant une résistance naturelle à la maladie sont connus actuellement et leur qualité de fruits (dattes) reste médiocre. Des méthodes conventionnelles et non conventionnelles sont en cours de développement et nécessitent une utilisation de techniques simple et pratiques pour cribler les individus résistants. L'utilisation des toxines excrétées par le pathogène en tant qu'agent sélectif pendant l'étape de culture in vitro devrait pouvoir augmenter la variabilité génétique et améliorer le niveau de résistance du matériel génétique. Foa produit des toxines telles que les acides fusarique, succinique et 3-phenyl lactique ainsi que leurs dérivés, mais également des marasmines et des toxines peptidiques. Ces toxines peuvent être utilisées conjointement ou séparément comme agents sélectifs. Cette contribution, qui constitue une revue sur l'utilisation des toxines comme agents sélectifs pour la résistance, tente d'amorcer une discussion à propos de l'utilisation potentielle de l'approche dans le cas du pathosystème palmier dattier–Foa. Cette revue ne prétend pas être ample ou exhaustive, dans la mesure où elle a été préparée dans le seul but de mettre en évidence l'utilisation potentielle des toxines excrétées par Foa dans la sélection in vitro de plants de palmier dattier avec un niveau de résistance acceptable. ***Pour citer cet article : A. El Hadrami et al., C. R. Biologies 328 (2005).***

Keywords: Date palm; Bayoud; *Fusarium oxysporum* f. sp. *albedinis* (Fao); Breeding for resistance; Toxins; In-vitro selection

Mots-clés : Palmier dattier ; Bayoud ; *Fusarium oxysporum* f. sp. *albedinis* (Fao) ; Amélioration génétique ; Toxines ; Sélection in vitro

1. Introduction

1.1. Overview

Host–pathogen interactions are governed by a complex of molecular and biochemical reactions, which ultimately result in the expression of disease resistance or susceptibility. In diseases caused by microorganisms producing toxins, these metabolites could be involved in the pathogenicity (the ability to cause disease) [1,2]. Toxins are produced by many microorganisms and are characterized by a high diversity in their chemical structure and physicochemical and biological properties [3]. Examples in the literature include diverse chemical classes including proteins, polypeptides, polyketols, terpenes, and/or glycoproteins [3–13].

The aim of this contribution is to give an overview on the use of toxins as selective agents and to highlight the potential use of this approach to the date palm–Foa pathosystem.

1.2. Progress in toxins characterization

Toxins produced by pathogens have been categorized based on several criteria, such as their chemical nature, mode of action, identity of the microorganisms producing them, and most definitions take into consideration their involvement as primary or secondary determinants of pathogenesis [2,14]. Toxins are primary determinants of pathogenesis when they act as the key element in infection initiation and symptom development. They are secondary determinants when they only modify the symptoms' intensity [14]. In order to assess the involvement of toxins in pathogenesis, commonly used criteria include (*i*) host-specificity [2,3,15–18], (*ii*) presence in infected plants [2,17,19–21], (*iii*) toxin production at a key step of disease development [2,21], (*iv*) induction of typical disease symptoms [2,22–25], and (*v*) degree of correlation between the quantity of toxin produced in-vitro and the pathogenicity level [2,15,17,26–28].

Several experimental approaches have been developed in order to evaluate the role of toxins in host–pathogen interactions [3,29]. The most convenient seems to be the inactivation or the specific elimination of the toxin from the system, followed by the observation of the modifications that occur during the initiation, establishment or expression of the host–pathogen interaction. For instance, antibody and metabolic inhibitors can be used for this purpose. However, these may not always have sufficient specificity to neutralize the action of a toxin [14]. Natural or induced mutagenesis that affects toxin production by the pathogen, or breeding that generates plant genotypes exhibiting resistance to the toxin, might also be used.

In spite of the interest that toxins represent in plant pathology, most attention has been dedicated to host-specific toxins and mycotoxins [30–34] rather than to the so-called non-host specific toxins, which affect a large spectrum of plant species [35,36].

1.2.1. Progress in the chemistry of toxins

Previous reviews have been published to detail the structure and the biochemistry of toxins [2,15,34,37–40]. Recent advances in isolation of genes have shown that genes required for toxin biosynthesis are unique to the toxin-producing species and are clustered in complex loci that may have been acquired by horizontal gene transfer [13]. Common mechanisms by which toxins arise from producing pathogens have then been suggested [13].

1.2.2. Toxins' mechanisms of action

Host-specific toxins have been stated to act directly on the plasmalemma, or on the mitochondria of sensitive cells [2]. This kind of toxins has been referred to as 'agents of compatibility' [39] and appears to mediate this compatibility by inducing host-cell death [3]. Host-specific toxins have been known, however, to act on the common plant metabolic process [5,16,17,20,23,40–42]. For example, the trichothecenes such as deoxynivalenol and 3-acetyldeoxynivalenol, non host-specific toxins, produced by both *Fusarium graminearum* and *F. culmorum*, inhibit eukaryotic protein synthesis by blocking the peptidyl transferase step [43]. It has been reported, but not convincingly proven, that plants tolerant to these toxins have a higher level of resistance to Fusarium Head Blight [44].

In host–pathogen interactions, toxins may block or attenuate expression of genes associated with an active resistance response and thereby decrease the ability of the plant to restrict colonization by the pathogen. Some 2-amino-8-oxo-9,10-epoxydecanoic acid (aoe) produced by fungi have been reported to interact with nucleic acids or proteins and exhibit cytotoxic effect that are associated with an inhibition of the macromolecular biosynthesis [45]. Chlamydocin, an aoe toxin produced by *Diheterospora chlamydosporia*, was shown to inhibit the thymidine incorporation into the DNA strand during the synthesis, leading to the restriction of the proliferation of the mouse mastocytoma cells grown in-vitro [46]. Trapoxin, another aoe toxin produced by *Helicom ambiens*, was reported as an anti-tumor agent that inhibits mammalian histone deacetylase and stop the cell cycle in cultured fibroblast cells [47]. Such effects were related, in both instances, to the epoxy group of aoe that can covalently bind the mycotoxins to the guanosine residues of the DNA and account for a carcinogenic and mutagenic agent in various animal species [48,49]. For plants, the most advanced studies on the effect of toxins on the DNA were carried out using the HC-toxin produced by *Cochliobolus carbonum* on maize [45]. The HC-toxin insensitive lines have a HC-toxin reductase that is able to reduce the ketone function to a hydroxyl group and inactivate the toxin [50]. Although the fact that this toxin was not found to be directly involved in the general processes of protein biosynthesis (e.g., amino acid uptake and translation; [45]), production of a number of specific proteins in maize was found to be affected in certain genotypes treated with the toxin [45]. Obviously, the regulation of the gene expression in toxin-treated susceptible cultivars of maize was influenced by the time and the concentration of the toxin applied and most importantly by the intact epoxy group of the molecule. Other host-selective toxins than the HC-toxin are known to affect the gene expression in susceptible host genotypes. Periitoxin, produced by *Periconia circinata*, was reported to up-regulate the expression of the gene(s) coding for specific charged isomers of a 16-kDa protein [51].

2. In-vitro selection for disease resistance

In-vitro techniques used for plants provide systems that are analogous to the prokaryotic systems, where mutations can be efficiently induced and variants selected and isolated at the cellular level. In-vitro selection represents an immediate and inexpensive way of generating/selecting plant variants with tolerance to either the pathogen or its toxin from susceptible varieties, as compared to classical crossing methods. Such techniques along with others may allow introgressing only desired traits to economically important varieties, without dramatically changing other desirable agronomical and resistance characters, as shown by Evans and Sharp [52]. Pathogens or their metabolites could be used as selective agents to enhance resistance within tissue cultures and to select resistant individuals.

2.1. In-vitro selection using pathogens

In-vitro selection to improve resistance against disease, using pathogens' spores as the selective agent, is a promising technique, particularly for pathogens not known to produce toxins. Such a direct method is useful for comparing responses of in-vitro plant material, known to be either susceptible or resistant to the pathogen, in order to determine if resistance is expressed at the cellular level. It is important to achieve such a selection in order to have a good understanding of the pathogen's life cycle and the conditions of its release. The first time that pathogen's spores were used to select resistant plant tissues was reported by Ingram [53,54]. Many other studies, particularly those of Helgeson et al. [55,56] have reported the formation of resistant calli from resistant plants of tobacco when they are challenged in vitro by spores of *Phytophthora parasitica*. Similar results, showing reduced growth of the pathogen on resistant calli, as compared to susceptible ones, and a correlation between the level of resistance to the pathogen of the cultured tissues and that of adult plants, were reported [57–59]. A correlation between the degree of resistance at the level of tissue growing in vitro and adult plant reactions was not usually confirmed. Moreover, and despite the efficiency of infection during tissue culture, it is not all the time possible to get selected lines carrying resistance. In most cases, this seems to be due to the loss by cultured tissues of their regeneration capacity [60,61].

Several selection attempts have been made using pathogens as selective agents and the majority involved viruses on protoplasts [62,63]. Protoplast infection has not been proved to be effective for selecting virus-free lines (resistant to the infection with the virus) because it was so difficult to obtain 100% of infection within protoplast cultures, and to distinguish between resistant protoplasts and those escaping the infection [62]. Varying results have been obtained when pathogens other than viruses were used as selective agents. Indeed, no resistant calli were obtained for *Brassicaceae* when they were selected under inoculation with spores of *Phoma lingam* and *Plasmodiophora brassicae* [60,64]. Contrarily, an increase in the disease-resistance frequency was observed within variants derived from celery tissue culture and grown on media containing *Fusarium oxysporum* f. sp. *apii* [65]. In the same way, Prasad et al. [66] have reported a regeneration of pearl millet plants resistant to *Sclerospora graminicola* from explants pre-infected by the pathogen.

The use of pathogens to select cells for disease-resistance, when used under the right conditions, could be efficient in some cases, and should be tried particularly in situations where no other selection procedure is available. In spite of the difficulty represented by the non uniformity of cell exposure to the pathogen with this approach, consideration should be also given to the expression of resistance under tissue-culture conditions [67].

2.2. In-vitro selection using pathogen's culture filtrates

This strategy is based on the use of more or less purified culture filtrates of the pathogen as a selective agent. This type of in-vitro selection has been first described by Behnke [68], who has used, in the absence of toxins derived from *P. infestans*, culture filtrates of this pathogen. Survival callus of potatoes have been obtained using a medium culture containing a pathogen filtrate concentration that is known to lead to 90% of mortality, and transferred many times on media containing culture filtrates. Regenerated plants using this system have exhibited reduced lesions when they have been challenged by the pathogen under greenhouse conditions comparatively to controls. Similar results have been reported in several pathosystems (Table 1). Some of these studies have shown a transfer of resistance from generated plants to their progeny [61,64,69], or a significant correlation between callus resistance to culture filtrates and resistance of the genotype to the pathogen [70,71].

The in-vitro selection using pathogen culture filtrates is much practical than that using the pathogen but it has a major inconvenient, which is that culture filtrates contain many non-determined toxins and the selected resistance could be for minor phytotoxic compounds of the filtrate or for cytosolic enzymes or the combination of the two. As a consequence, results should be compared with those using purified toxins in order to discard selection for components that are not essential.

Table 1
Several examples of in-vitro selection using pathogen culture filtrates

Pathogen	Host	Selected material	References
Phytophthora infestans	*Solanum tuberosum*	Callus	[125]
Fusarium oxysporum	*S. tuberosum*	Callus	[126]
Phoma lingam	*Brassica napus*	Callus	[64]
Alternaria alternate	*Nicotiana tabacum*	Callus	[68]
Pseudomonas syringae	*N. tabacum*	Callus	[69]
F. o. f. sp. *medicaginis*	*Medicago sativa*	Callus	[127,128]
F. o. f. sp. *dianthi*	*Dianthus caryophyllus*	Callus	[129]
Helminthosporium sativum	*Hordeum vulgare*	Callus	[79,80]
H. sativum	*H. vulgare*	Embryo-derived callus	[85]
F. o. f. sp. *asparagi*	*Asparagus officinalis*	Callus	[130]
Stagnospora nodorum	*Triticum aestivum*	Zygotic embryos	[131]
F. o. f. sp. *subglutinans*	*Ananas comosus*	Callus	[122,123]

2.3. In-vitro selection using more or less purified pathogen toxins

Studies of toxins involved in diseases were of interest for practical and conceptual reasons [2]. Practically, their use simplifies greatly the biochemical analyses of the interaction because they act as reliable surrogates for the pathogens producing them. Conceptually, the early pre-treatment of plant tissue-cultures with toxins or culture filtrate containing toxins have shown that tissue response in-vitro correlates often with disease reaction of the hosts. Moreover, such treatments might increase in some cases the level of resistance against several pathogens [72–74].

Tissue-culture techniques have produced *germplasm* with enhanced disease resistance [73,75]. The availability of a defined toxin is advantageous for developing in-vitro selection protocols. Although these substances often have phytotoxic properties, their possible roles in the pathogenesis have not been thoroughly investigated because it is difficult and time-consuming [3,76]. However, before even attempting to exploit such compounds for breeding purposes, their role in plant pathogenesis has to be assessed. To be useful, toxins must be involved in the disease development process, act at the cellular level, and have a mode of action that allows recovery of resistance [73,75].

By applying pathogen toxins for isolation of disease-resistant cell lines, the efficacy and efficiency of induction of nucleic or cytoplasmic viable-alterations and identification of useful mutants could be increased considerably. The idea of using a toxin produced by a pathogen as a tool for resistance breeding dates from over 30 years ago and was used in many studies and several examples of *germplasm* screened for disease resistance using a toxin as discriminating selective agent are well documented [73,77]. Indeed, pathogen-resistant mutants of rice, tomato and oat have been isolated and used in breeding programmes in several countries using this approach (Table 2). For example, fusaric acid produced by many *formae specialis* of *Fusarium oxysporum* has been used as a selective agent to select tolerant callus of tomato, barley, pears, and melon [70,74,78–82].

The use of toxins in screening for resistance may present many difficulties. A major problem is the discrepancy that is often observed between screening results obtained in the field and those obtained under laboratory or greenhouse conditions. Other encountered problems are of three levels: (*i*) the lack of sufficiently characterized toxins with a proven role in the disease, (*ii*) the variation of single cells vs. whole plant resistances, and (*iii*) the dependency of the mode of action of the used toxin.

3. Experimental protocols for selection using toxins

3.1. The use of toxins to increase host variability

The sources of resistance available in nature are being exhausted and there is a need to increase genetic diversity in commercial varieties. The induction of genetic variability and the selection for improved gene

Table 2
Several examples of in-vitro selection using pathogen toxins

Pathogen toxins	Host/pathogen interaction	Selected material	References
Methionine sulfoximine	*Pseudomonas tabaci*/*Nicotiana tabacum*	Callus	[72]
T-Toxin	*Helminthosporium maydis* race T/*zea mays*	Callus	[114,132]
Helminthosporoside	*H. sacchari*/*Saccharum officinarum*	Callus	[133]
Cercosporin	*Cercospora* sp./*Nicotiana* sp.	Callus	[134]
Victorin	*H. victoriae*/*Oryza sativa*/*Avena sativa*	Callus	[78,135]
Fusaric acid	*Fusarium oxysporum* f. sp. *lycopersici*/*lycopersicum esculentum*	Callus	[78,136]
Fusaric acid	*Fusarium* sp./*Hordeum vulgare*	Callus	[79,80]
Fusaric acid	*F. o.* f. sp. *gladioli*/*Gladiolous* sp.	Cell suspensions	[74]
Sirodesmine	*Phoma lingam*/*Brassicaceae*	Callus	[137]
Sirodesmine	*Myrothecium roridum*/*Cucumis melo*	Callus	[81]
Tenuazoic acid	*Magnaporthe grisea*/*Oryza sativa*	Protoplast-derived callus	[136]
Roridin	*Cucumis melo*/*Myrothecium roridum*	Callus	[81]

combination among variants are presently considered to be important aspects of plant breeding. Furthermore, nuclear irregularities have long been known to occur in tissue culture cells [83,84], and phenotypic abnormalities occur fairly commonly in regenerated plants. One approach would be to use toxins for in-vitro selection of insensitive cells and subsequent regeneration of resistant plants [79,80,85–87].

3.2. The use of toxins as early screening tools of newly created genetic material

In order to decrease the amount of field inoculations, studies were carried out in many plant–pathogen systems to develop a simple laboratory method to examine resistance of breeding material. In the literature, several methods were described to select plants with increased level of disease resistance using toxic metabolites produced by pathogens as selective agents [73].

Insensitivity to phytotoxins produced by plant pathogens can be used as a marker for the early screening of segregating populations within conventional and non-conventional breeding programmes as reported in many host–pathogen systems [88,89]. This approach requires some knowledge about the correlation between sensitivity to toxins and susceptibility of plants to the pathogens that produce them. Such correlation need to be highly significant, even if the goal is to select resistant plants within a long-term breeding program.

4. What about the application of the approach in the case of *Phoenix dactylifera* L.–*Fusarium oxysporum* f. sp. *albedinis* system?

4.1. The date palm

The date palm (*Phoenix dactylifera* L.) belongs to the *Arecaceae* (*Palmaceae*) family, which contains over 200 genera and over 2500 species. It is a long-lived monocotyledon species with a dioeciously character (unisexual inflorescences were carried by two individuals that can be male or female) and it is represented by almost 100 million of trees throughout the world. About 66% are located in Asia, 32% in Africa, 0.5% in California and Mexico, and 0.3% in Europe.

The species has been largely propagated by offshoots and by seeds. Offshoots show a slow development and their number is not sufficient for the large scale propagation. A selected tree can develop 0 to 3 offshoots per year and not more than 10 to 40 during its lifetime depending on the cultivar and the environmental conditions. Such a characteristic represents a great limiting factor for traditional propagation of the species. Moreover, seedlings issue from seeds are hybrids and do not have the same mother characters. This propriety makes complicated the optimisation of the conventional breeding program. Flowering and fruiting are very slow and it is difficult to determine the sex of the trees before the first flowering, which arises about five years after the planting. To overcome such difficulties, the development of biotechnologies to increase the plant production using in-vitro culture prop-

agation was so promising but it had not yet got a great success taking into account the complexity and the time needed for conducting date palm tissue culture.

Date palm is originating from aridic and Saharan countries, and is of a major ecological and socio-economical importance, especially in the north of Africa and in the Middle East [90]. Its contribution to the statement of the ecophytocenose of oases, and the creation in the middle of the huge desert of a microclimate favourable for vegetable growth and consequently a surviving source for humans and their animals, is of importance. Indeed, the maximum of vegetative growth of date is reached at high temperatures (30–40 °C). Date palm is also a source of production of an appreciated fruit (dates). The production of dates remains related to the genotype and the environmental conditions, especially the availability of water, because paradoxically such desert 'tree' needs large quantities of water. For example, the production in natural conditions varies between 18 kg/tree/yr in Morocco and 50 kg/tree/yr in Tunisia. In Morocco, and according to the rainfall, the production was about 12 000 tons during a dry year (1985), while it reached 120 000 tons during a wet year (1990).

4.2. *The date palm-fusariosis wilt, the bayoud*

Unfortunately, date palm patrimony was decimated since several decades and continues to be attacked specially in Morocco and Algeria by the bayoud disease. This detrimental vascular disease is caused by *Fusarium oxysporum* f. sp. *albedinis*. It causes withering then decays of the date palm trees and it constitutes a major constraint for the phoenicicole regions in the north of Africa, particularly for those not presenting yet the disease, notably Tunisia [90,91]. The bayoud was firstly described in Morocco in 1870 [92], then in Algeria [90,93,94]. Since the date palm-fusariosis wilt epidemic still in spread, almost all the plantations in Morocco and an important part of plantations in western and central Algeria are infested, resulting in death of several millions of trees. The estimated destroy is about two thirds of the Moroccan date palm plantations (10 million trees), resulting in the progressive disappearance of the good cultivars from the genetic stock in Morocco. Moreover, fusariosis is still extending in Algeria, where several millions of trees have been hit and threat the Tunisian date palm plantations [91,95].

The disastrous impact of bayoud is not only that it contributes to a fall in date production, a staple food for both humans and livestock in the Sahara desert, but is also upsetting the oasis ecosystem considering the environmental role of date palm there. Indeed, this tree acts as a windbreak and protects oases from desert encroachment and it creates a microclimate favourable for cultivation of other crops (fruit trees, cereals, fodder crops and market garden products) essential for the oases people and their live-stocks.

4.3. *The* Fusarium oxysporum *f. sp.* albedinis *species*

Fusarium oxysporum f. sp. *albedinis* (Foa) is the causal agent of the date palm bayoud wilt. This deuteromycete species is a soil-borne pathogen that seems to be adapted to hostile conditions in soil in the absence of its host until the optimal conditions of spread. No known sexual form was described for this pathogen.

Pathogen population structures both in Morocco and Algeria were studied using molecular markers [96,97]. Indeed, the existence of different lineages of the pathogens have been demonstrated, which might lead to a quick overcome of selected resistances. Furthermore, inoculation tests have revealed a variability of the pathogenicity level of Foa. Propagation of the pathogen is mainly due to the genetic material exchange between the two countries, which had occurred by the past, and in the same oases between infected roots. Diagnostic tools of Foa have been established using the PCR amplification of *Fot1*-like transposing elements [97]. This method is already used for monitoring the disease in date palm plantations, for controlling plant material designated for exportation and also for date-palm improvement program.

The examination of bayoud symptoms suggests an involvement of at least one toxin in the Foa pathogenesis mechanisms [98]. Indeed, Foa excretes several toxins such as fusaric, succinic, 3-phenyl lactic acids and their derivatives [6–8,99] and peptidic toxins [6–8,99]. Up to date, no study has been conducted to understand the mechanism of action of these toxins. Only few studies have been initiated to screen different strains of the pathogen for their production of marasmins, fusaric, succinic and 3-phenyl lactic acids, and other toxic peptides [6–8,99–102] and to characterize different peptidic fractions supposed to be toxic

against date-palm seedlings [6–8,99]. Complementary studies are needed to dissociate the effect of different toxins on date palm and to set up a protocol using them bulked or separately to analyze their respective role in Foa pathogenesis.

4.4. Developed strategies to control the date palm bayoud wilt

Taking into account the vascular character of the bayoud disease, most control strategies were those recommended for other fusariosis and particularly for those applied to perennial species. Indeed, such strategies could be confined to prophylactic measures and cultural practices, which lead to a delay in the progression of the bayoud, but not to its eradication. Breeding for resistance to bayoud is the most promising strategy, because it presents an efficient and economical way to fight this disease. Both incompatibility (complete resistance supposed monogenic to oligogenic with a hypersensitive-like reaction) and compatibility (partial resistance presumed polygenic) reactions have been described within the date palm–Foa pathosystem [98]. Taking into account the genetic diversity of the pathogen and its variation in aggressiveness, breeding for partial resistance might be more durable.

Breeding of date palm, developed in Morocco (the endemic bayoud country) since 1963, was first based on the screening of natural genotypes for their resistance against the bayoud and then on the creation of new hybrids carrying suitable levels of resistance and a good quality of fruits. Among 223 Moroccan natural varieties of date palm described and tested for their resistance to bayoud, six, including Bousthami Noire, Bousthami Blanche, Iklane, Sair-Layalate, Tadment and Bouffegous-Oumoussa, have shown a potential for resistance to bayoud in spite of their mediocre quality of dates [90,103–105]. Commercial varieties with higher quality of dates, introduced from Irak or Tunisia, have exhibited, however, a high susceptibility to Foa [105–107]. The use of controlled cross has led to the selection of some genotypes carrying both desirable agronomic characters and an acceptable level of resistance to bayoud [91,105,107]. The development, in the last decades, of in-vitro techniques such as organogenesis and/or somatic embyogenesis [99, 108–111] has greatly contributed to increase the production of potential candidates for overcoming or reducing the impact of the bayoud disease. Such techniques might be used also to extend or to restore the genetic pool, which has been impoverished by discarding cultivars of medium and low quality of dates from the oases plantations. The human selection has led in many countries to reduce the genetic diversity grown in the field, as reported by Al Khalifah and Askari [112] in Saudi Arabia. This expansion of the monovarietal cultures will have harmful repercussions in the future [113]. Thus, the cultivar ‘Deglet Nour’ occupies currently 45% of the Algerian palm groves and approximately 60% of those in Tunisia. Moroccan groves still have some diversified date palm plantation called ‘Khalt’.

4.5. The potential use of toxins within the date-palm breeding program

Genetic improvement of date palm based on conventional and non-conventional breeding schemes need to use the simplest and easiest methods to screen resistant individuals. Generally, the use of toxins as selective agents at the tissue culture step represents one way of increasing variability and improving resistance of the genetic material [72–74,114,115]. In-vitro products of date palm such as callus, cell suspensions, protoplasts and somatic embryos could be challenged with culture filtrates, fusaric acid or more or less purified toxic peptides. Two objectives could be considered then: (*i*) inducing/increasing the genetic variability within tissue culture and (*ii*) early screening of new hybrids, produced within the breeding program, for their level of tolerance to toxins instead of the fastidious and time-consuming field assessment. To achieve the first objective, selecting callus, cell suspension, protoplasts or somatic embryos that could survive to increasing levels of toxins could be conducted. The correlation between the insensitivity levels of a referential set of seedlings to culture filtrates or to more or less purified toxins and the levels of resistance to the pathogen under controlled conditions should be investigated. In case of high correlations between this insensitivity at the cell level and resistance at seedling stage, culture filtrates or purified toxins could be used as selective agents and for early screening of hybrids produced through the breeding program.

Several studies have shown that even if it produces fusaric acid and marasmins, Foa can secretes also

some peptidic toxins [99]. These toxic peptides have been separated using semi-preparative HPLC in three fractions and then tested on detached leaves of date palm seedlings allowing the determination of their individual phytotoxicity and their synergistic actions. In addition, a system, based on the callus reaction on the culture filtrate of Foa, suitable for studying date palm–Foa interaction has been described recently by our group [116,117]. With this useful model system, it is possible to distinguish between resistant and susceptible cultivars according to their response in terms of biosynthesis and accumulation of phenolic phytoalexins [118–120]. These first works have also shown that the response of the whole plant to the fungus invasion can be found at the cellular levels (embryogenic cell suspensions and somatic embryos) using Foa culture filtrates (unpublished data). The observed elicitation of defence reactions might be due to the exogenous elicitors secreted by the pathogen into the culture medium. We can speculate then that toxic peptides of Foa could act as the main elicitors of defence mechanisms and would be useful as selective agents of date-palm resistant material.

5. Criticism to the approach using toxins in selection schemes

Despite the apparent popularity and usefulness of the approach using toxins for in-vitro selection of resistant lines, no resistant varieties have been yet generated in this way, even though not enough material has been generated for many pathosystems or the regenerated plants have shown abnormal functioning [73,74, 121]. Furthermore and even though Daub [73] has expressed some concerns about using pathogen metabolites as selective agents to screen tissue culture for resistance, application of phytotoxins has been proposed for in-vitro screening of many plant systems [74,115, 122,123] (Table 2). Many works aiming at producing resistant genotypes by selecting callus that survives to increasingly higher concentrations of toxins have been conducted [124]. Nevertheless, the increase of the level of insensitivity to toxins did not often lead to an increase in the resistance to the pathogen.

From the plant pathological point of view, selecting resistant plant material using one molecule would lead to results similar to those obtained by selecting on a single gene basis. However, including such an approach in a complete breeding program to increase variability within available genetic material should be appreciable.

6. Concluding remarks

Production of date palm individuals carrying suitable levels of resistance against bayoud using toxin-based in-vitro selection might be of interest for the breeding program. Further studies to understand the toxin's mechanism of action, the degree of involvement in pathogenicity have to be conducted to insure the objectives of the selection program. Focus goals might then be defined and different ways of using toxins might be explored.

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