**MIRELLE NAYANA DE SOUSA SANTOS**

# INHIBITION OF POTATO TUBER SPROUTING AND INDUCTION OF DRY ROT RESISTANCE DURING STORAGE IN RESPONSE TO 1,4 DIMETHYLNAPHTHALENE AND METHYL JASMONATE

Thesis submitted to the Plant Physiology Graduate Program of the Universidade Federal de Viçosa in partial fulfilment of the requirements for the degree of *Doctor Scientiae.*

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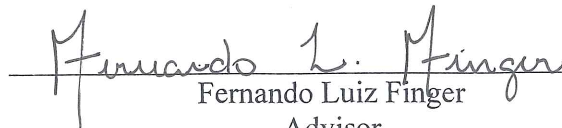

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Advisor

*To Paula Lima for the friendship and partnership in this work (in memoriam).*

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# ABSTRACT

SANTOS, Mirelle Nayana de Sousa, D.Sc., Universidade Federal de Viçosa, May, 2021. **Inhibition of potato tuber sprouting and induction of dry rot resistance during storage in response to 1,4 dimethylnaphthalene and methyl jasmonate.** Adviser: Fernando Luiz Finger. Co-advisers: Marcelo Rogalski and Ana Maria Mapeli.

Potato is globally the third most important food crop and one of the most consumed foods in the world. The Brazilian production is traded either *in natura* or processed, which requires long-term storage to keep a continuous on-demand supply. One of the biggest challenges regarding potatoes production system is the complexity in delaying the tuber physico-chemical changes during storage. Alongside, the major factors related to excessive costs into potato production are the significant losses associated with the tuber's susceptibility to fungal diseases and the intense sprouting incidence after the natural dormancy breakdown. Several techniques have emerged to prolong the interval of potato tubers storage, and this includes the addition of chemicals associated with a cooling system. However, issues related to human toxicology and/or environmental concerns have fostered studies on both non-toxic sprouting inhibitors and plant resistance inducers for tubers. Dimethylnaphthalene (DMN) presents inhibitory properties related to sprouting growth, however, the mechanisms by which DMN underlies other biological effects are unknown. Likewise, Methyl Jasmonate (MeJa) has been considered a sprout inhibitor and a potential resistance inducer. Based on this, the present study investigated the mechanisms induced by DMN and MeJa application underlying both sprouting inhibition and resistance induction to dry rot (*Fusarium nirenbergiae*) by assessing physiological, physico-chemical, and molecular parameters in stored Asterix and Challenger potato tubers. DMN and MeJA reduced the incidence of sprouting and fresh weight loss, and decreased the effects of reducing sugars on non-enzymatic browning and altered the expression of KRP family cell cycle inhibitor genes in Asterix. However, the treatments did not affect the expression of enzymes related to antioxidant and defense systems in both Asterix and Challenger potato tubers. The efficiency of DMN in delaying dry rot incidence caused by *F. nirenbergiae,* both *in vitro* and *in vivo*, is not related to changes in the activity of antioxidant and defense-related enzymes, which suggests a likely direct action of the compounds on the pathogenicity. Moreover, the results evidence a certain level of resistance in Asterix and Challenger cultivars.

Keywords: *Solanum tuberosum*. Sprout inhibitors. Non-enzymatic browning. Endodormancy. Cell cycle inhibitors. Postharvest diseases. Induced resistance.

# RESUMO

SANTOS, Mirelle Nayana de Sousa, D.Sc., Universidade Federal de Viçosa, maio de 2021. **Inibição da brotação e indução de resistência a podridão no armazenamento da batata em resposta ao 1,4-dimetilnaftaleno e metil jasmonato**. Orientador: Fernando Luiz Finger. Coorientadores: Marcelo Rogalski e Ana Maria Mapeli**.**

A batata é a terceira cultura alimentar mais importante do planeta, estando entre os alimentos mais consumidos no mundo. A produção nacional é comercializada in natura ou na forma de processados, o que requer um abastecimento contínuo e um armazenamento de longo prazo após a colheita. No entanto, o maior obstáculo ao uso da batata é a dificuldade em retardar alterações pós-colheita. Dentre os diferentes fatores envolvidos, estão as grandes perdas devido à susceptibilidade dos tubérculos a doenças fúngicas e a intensa brotação após a quebra natural da dormência. Diversas técnicas têm sido utilizadas com o objetivo de aumentar o período de armazenamento dos tubérculos, incluindo a adição de produtos químicos associados à refrigeração. Contudo, questões relativas à toxicologia e aos efeitos sobre o meio ambiente vem estimulando pesquisas com inibidores de brotação e indutores de resistência não tóxicos em tubérculos. O 1,4 Dimetilnaftaleno (DMN) possui propriedades inibidoras relacionadas ao crescimento de brotos, entretanto, os mecanismos pelos quais o DMN exerce outros efeitos biológicos são pouco explorados. Da mesma forma, o Metil Jasmonato (MeJa), em estudos prévios é considerado um inibidor de brotação e potencial indutor de resistência. Portanto, objetivamos investigar os mecanismos induzidos pela aplicação do DMN e MeJa na inibição da brotação e indução de resistência a podridão seca causada por Fusarium nirenbergiae, através da avaliação dos efeitos fisiológicos, físico-químicos e moleculares em tubérculos de batata cultivares Asterix e Challenger armazenados. O DMN e MeJA reduziram a incidência de brotação, perda de massa, minimizaram os efeitos dos açúcares redutores no escurecimento não enzimático e alteraram a expressão dos genes KRP envolvidos no processo de regulação do ciclo celular em tubérculos de batata Challenger. Entretanto, ambos os tratamentos não alteram significativamente a expressão das enzimas relacionadas com o sistema antioxidante e de defesa em tubérculos de batata Asterix e Challenger. A eficiência do DMN em retardar in vitro e in vivo a infecção da podridão seca causada por F. nirenbergiae, não está relacionada com alterações nessas enzimas, o que sugere uma possível ação direta dos produtos sobre o patógeno e não no tubérculo. Ademais, os resultados encontrados confirmaram o nível de resistência presente nas cultivares Asterix e Challenger**.**

Palavras-chave: *Solanum tuberosum*. Inibidores de brotação. Escurecimento não enzimático. Endodormência. Inibidores do ciclo celular. Doenças Pós-colheita. Indução de resistência.

## SUMMARY

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# GENERAL INTRODUCTION

Potato (*Solanum tuberosum* L.) is the third globally most produced food crop and the principal non-grain commodity (Embrapa, 2018) - standing among the most consumed foods on the planet. In Brazil, potatoes are a very important staple food, with an annual production of approximately 3.7 million tons in an area that covers 117 thousand hectares (IBGE, 2020), in which the total production is estimated to increase about 3.5% by 2021, according to the 2020/2021 Hort-fruit Brazil Journal Yearbook. The state of Minas Gerais stands out as the largest potato producer in the country, with favorable edaphoclimatic conditions. In this state, potato production occurs over the year, which generates jobs and incomes in different sectors from crops to industrial processing.

Domestic production has been traded either *in natura* or processed, such as chips, fries, and by-products, which require a continuous supply throughout the year and longer postharvest storage. Among the different factors involved with potato production costs are the fungal disease occurrences, such as late blight (*Phytophthora infestans*), black spot (*Alternaria* spp.), dry rot (*Fusarium* spp.), among others. When not properly controlled, they may cause either total loss of production or affect the postharvest overall quality (Fioreze, 2003).

Fourteen species of *Fusarium* have been worldwide closely related to dry rot in potatoes (Gachango et al., 2012). Other pathogenic *Fusarium* species associated with potato dry rot include *F. coeruleum*, *F. avenaceum*, *F. culmorum*, *F. oxysporum*, and *F. graminearum* (Hide et al., 1992; Vaughn and Spencer, 1994; Hanson et al., 1996; Peters et al., 1996; Peters et al., 2008; Bojanowski et al., 2013), being one of the most relevant diseases in potato tubers during the storage period, causing production losses, thereby affecting up to 60% of the tubers. This pathogen is common in most of the soils that potatoes are grown and survive as resistant spores in both soil and plant tissue (Hooker 1981). Although the tuber infection may occur before harvesting, the lesions are severe when the fungus infects through wounds caused mainly by handling in harvesting, sorting, and storage (Dean, 1994).

Currently, the preventive control strategies for dry rot include the use of resistant cultivars and cultural practices such as the use of healthy seeds and wound healing during the curing process before storage. Resistance is a key element in strategies to control dry rot, however, cultivars vary in their level of resistance to *Fusarium* spp. Furthermore, none have yet been presented a full resistance to all *Fusarium* species (Corsini and Pavek, 1986). Among the potatoes produced in Brazil concerning industrial processing are the Challenger and Asterix cultivars, which present a higher fitting and yield and good storage capacity (Hzpc, 2021),

besides a suitable market acceptance. However, Challenger has been shown a higher degree of susceptibility to dry rot.

In addition to preventive strategies, the curative control can be performed by using chemicals as fungicides. However, due to concerns regarding pathogen resistance to chemicals and potentially harmful effects on the environment and human health, current production systems require crop protection through both innovative eco-friendly compatible methods and sustainable agriculture instead of a chemical application purely (Kuc, 2001; Tian and Chan, 2004).

An emergent method to the management of such diseases is based on the induction of natural defense mechanisms via resistance inducers application. This technique has been widely studied and requires elucidation on plant defense mechanisms to elicit such events without affecting growth and yield (Walters and Boyle, 2005). In this sense, plant-induced resistance via the elicitor has been considered a promising way for disease management (Bi et al. 2006).

Concomitantly, severe sprouting after natural breaking dormancy adversely affects the potato's nutritional quality as well as processing characteristics during storage (Finger et al., 2005). After harvesting, potato tubers are dormant and do not emerge even whether placed under suitable conditions. This latent state can be affected by both the pre-and post-harvest environment (Suttle, 2004). Moreover, tuber dormancy life span also differs among cultivars and is influenced by the harvest time and tuber’s maturity status before storage (Hay and Porter, 2006).

Tuber breaking dormancy and shoot growth are accompanied by biochemical changes (Suttle, 2004), as the sprouting mobilizes starch and consumes part of the tuber's energetic reserves, leading to increased sugar content, weight loss, and wilting (Finger et al., 2005).

The mechanisms underlying breaking dormancy and sprout growth are not yet clear, as are the related-molecular bases (Sonnewald and Sonnewald, 2014). Depending on purposes, an accelerated sprout growth (production of seed potatoes) or delayed development (industrial processing) is favorable. To prolong the tuber dormancy timeframe, the tubers are stored at low temperatures or treated with sprout inhibitors/suppressors. However, low temperatures induce starch breaking into sugars, a phenomenon known as cold-induced sweetening. This process reduces the tuber quality, especially when destined to the industry (Sonnewald, 2001).

Several techniques have been used to increase the tuber's storage period, including the addition of chemical products associated with refrigeration. Chloropropham (CIPC) is a chemical inhibitor used to reduce the development of sprouts by one application, which provides the extension of the tuber's shelf life and reduced dehydration (Kleinkopf et al., 2003;

Frazier et al., 2004). However, after severe restrictions in Europe, it is only registered for use in potato storage in the United States, not being registered for potatoes in Brazil. In addition, issues related to toxicity and environmental effects have been raised by health and environment agencies, thereby leading to a significant reduction in the use of CIPC for such purposes (Goméz-Castilho et al., 2013). Therefore, alternative methods for controlling tuber sprouting during storage are needed.

1,4-Dimethylnaphthalene (DMN) is a natural compound present in potato tuber tissues with a well-known ability in inhibiting sprout growth (Campbell, 2010). It has been synthetically produced for use as a plant growth regulator. Specifically, the substance inhibits sprout formation in stored potatoes, thus prolonging effective storage time, maintaining the tuber overall quality (Campbell, 2012). Given recent reductions in the tolerance of CIPC residues in potatoes, a growing interest in the application of DMN to control worldwide sprouts during storage has been renewed (Kleinkopf et al., 2003). In addition to its property in controlling sprouting in potatoes for both processing and fresh, the reversible effect of naphthalene also enables its use in potato tuber seeds.

Although the inhibitory properties related to sprouting growth as well as the commercial utilization of DMN have been well-known and explored over several years, the mechanisms by which DMN exerts other biological effects are poorly understood. Preliminary studies have observed reductions in the qualitative losses of potatoes during storage after the application of DMN, which was closely associated with the lower incidence of fungal pathogens, thereby indicating a likely fungicidal property. However, it is unclear whether a decreased rate of disease is a direct response to DMN on the pathogen or whether it leads to an induced resistance to this disease. Analysis of gene expression in DMN-treated potato tubers showed significant increases in the PR4 and PR5 genes associated with the pathogenic response (Campbell et al. 2016). However, the DMN way of action as an inhibitor of sprouting and its effectiveness as a probable inducer of resistance in commercial potato varieties grown in Brazil needs to be investigated.

Jasmonates are a group of compounds widely known for triggering many physiological and biological responses in several plant species (Wasternack, 2007), but information about their role in sprouting control is still incipient (Allah et al., 2018). According to the patent by Lulai et al. (1995), the treatment by immersion in methyl jasmonate (MeJa) emulsions at concentrations ranging from 1.0 to 10 μM delayed the beginning of sprout growth in freshly harvested potatoes, with an effect similar to that of CIPC. Furthermore, there was a significant

improvement in the color of potato chips. Concomitantly to DMN, MeJa's mechanism of action in controlling sprouting in potato tubers remain unknown.

Moreover, exogenous application or inductions of endogenous resistance organic acids synthesis, such as methyl jasmonate (MeJa) derived from jasmonic acid, can act as inducers of tolerance proteins to different stresses, as well as elevating enzyme activity of cell detoxification, especially those involved with the elimination of reactive oxygen species (ROS) (Genzel, et al., 2018; Nascimento, et al., 2015). Therefore, they can act as resistance inducers against several pathogens. Studies have demonstrated a reduction in infection via systemic transcriptional induction of defense-related genes associated with jasmonic acid signaling pathways in potatoes infected with *Rizoctonia solani* AG3PT (Genzel et al. 2018), highlighting its potential as a resistance inducer also in specific organs of plants, like tubers.

Therefore, the use of non-toxic sprout inhibitors and resistance inducers has stimulated studies aimed at understanding the biochemical and molecular aspects involved with sprouting qualitative reduction and promoting disease resistance. Therefore, we aim to investigate the mechanisms induced by the application of 1,4-Dimethylnaphthalene (DMN) and Methyl Jasmonate (MeJa) in the inhibition of sprouting and induction of resistance to dry rot caused by *Fusarium nirenbergiae*, a *Fusarium oxysporum* complex pathogen, by evaluating physiological, physicochemical and molecular effects on potato tubers of Asterix and Challenger cultivars stored at 8 °C.

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# CHAPTER 1

## Inhibition of potato tuber sprouting during storage in response to 1,4-dimethylnaphthalene and methyl jasmonate

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## Inhibition of potato tuber sprouting during storage in response to 1,4 - dimethylnaphthalene and methyl jasmonate

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## ABSTRACT

Only a few inhibitors are used to control sprouting during potato storage. Currently, issues related to the storage of tubers have taken on a new importance, due to the toxicity of the main

sprout inhibitor, chloropropham (CIPC). This work evaluated the action of 1,4-dimethylnaphthalene (DMN) and methyl jasmonate (MeJa) on tuber sprouting cv Challenger stored at 8 °C. DMN and MeJa were fogged for 2 h onto followed by 90 days of storage. Fresh mass loss, the incidence of sprouting, sprout length, carbohydrate metabolism, and French fry color were determined. The expression of KRP1 and KRP4 genes was also determined. DMN and MeJA reduced the incidence of sprouting, mass loss by the tubers, minimized the effects of reducing sugars on non-enzymatic browning and changed the expression of the KRP genes involved in the process of cell cycle regulation. Therefore, DMN and MeJa can be used to inhibit potato tuber sprouting without the toxicity of CIPC.

*Keywords:*

*Solanum tuberosum*

Non-enzymatic browning

Endodormancy

Cell cycle inhibitors.

## 1. Introduction

Most of the potato supplied to the processing industry comes from long-term storage. Potatoes have a natural dormancy and once they start sprouting, the physiology changes and causes remobilization of starch, and increased fresh mass loss and respiration. These changes result in the depreciation of the processed products (Sonnewald and Sonnewald, 2014).

Among the cultivars planted in Brazil, Challenger is well adapted to the temperature growing conditions and has quality standards required by the processing industry and Brazilian consumers. Tubers for industrial processing should have high dry matter content, low content of reducing sugars, and free of brown spots.

Tuber dormancy is genetically determined but is significantly affected by pre- and pos-harvest environmental conditions (Suttle et al., 2016). Since the end of the tuber dormancy period is characterized by the beginning of fast growth of the buds, delaying the end of dormancy is critically important to the potato industry. Thus, the control of storage temperature and chemical treatments methods are used to delay sprouting. Current methods to inhibit sprouting, however, have their limitations.

Because to the toxicity of the main sprout inhibitor, CIPC, there is an urgent search for new products that are less dangerous to the environment and to human health (Teper-Bamnolker et al., 2010). Therefore, the need for alternatives to control sprouting during long-term storage, is a priority to the potato processing industry around the world (Alamar et al., 2017). 1,4-Dimethylnaphthalene (DMN) is a natural volatile compound isolated from potato tubers that is effective in replacing CIPC (Beveridge et al., 1981). DMN temporarily prevents potato sprouting, making it an attractive sprout inhibitor for both seeds and processing-oriented tubers (Lewis et al., 1997). Contrarily to CIPC, DMN can be used to control sprouting in seed potato, increasing the yield and uniformity of plant emergence and establishment (Knowles et al., 2005).

Although the commercial utility of DMN has been known and exploited for several years, the mechanisms by which DMN exerts its biological effects are still poorly understood. Previous research has shown that DMN does not exert its activity in prolonging the natural tuber dormancy period by changing the hormone profile (Campbell et al., 2010). Campbell et al. (2012) showed that the inhibitory properties promoted by DMN are related to a disruption of cell cycle progression that results in inhibition of shoot cell division. In addition to the temporary effect in inhibiting sprout cell division, it is known that DMN also affects the expression of genes associated with the pathogenic response (Campbell and D'Annibale, 2016). Cell cycle inhibitors *KRP1* and *KRP2* have their expression altered by DMN (Campbell and

D'Annibale, 2016); never theless the action of MeJa on their expression has not been previously studied.

Jasmonates are a group of compounds known to elicit diverse physiological and biological responses in a wide range of plant species (Wasternack, 2007), but information on the role of jasmonates in controlling sprouting in potatoes is still scarce (Allah et al., 2018). According to a patent registered by Lulai et al. (1995), treatment by immersion in MeJa solution at concentrations varying from 1.0 to 10 μM retarded the onset of sprout growth in freshly harvested potatoes. In addition, there was a significant improvement in the color of potato chips. Concomitantly with DMN, the mechanism of action of MeJa to control sprouting in potato tubers remains to be completely known.

In the present study, we compare the effectiveness of DMN and MeJa to inhibit sprouting of potato tubers, the changes in sugar metabolism, and their action on *KRP* genes expression during storage of cv Challenger.

## 2. Material and methods

### *2.1. Plant material*

Tubers of cv Challenger grown in Perdizes, Minas Gerais, Brazil (1100 m a.s.l.,19º21'S, 47º17'W) were harvested from dead vines 120 days after planting. Disease-free tubers of 150 - 200 g were selected and allowed to wound heal for 7 d at 14 °C and 90 % relative humidity. Afterward, the temperature was reduced to 8 ºC for long-term storage.

### *2.2. Treatment with 1,4-dimethylnaphthalene (DMN) and methyl jasmonate (MeJa)*

Immediately after wound healing, tubers were treated with 20 mg DMN or 10 μM MeJa per kg of potatoes applied as vapor in 90 L sealed flask as described by Vaughn & Spencer (1991). Each flask contained 25 tubers and a Petri dish with DMN or MeJa in 3 mL of 95 % ethanol on a hot plate to vaporize the compounds. After 2 h, the tubers were stored at 8 °C and

90 ± 5 % RH. Control tubers were treated with vapors of 95% ethanol for the same period. After dormancy breaking, one additional application of DMN and MeJa was performed, following the procedure previously described.

*2.3. Tuber loss of fresh mass, number of sprouts, and length of sprouts*

Throughout storage, tubers were weighed to estimate accumulated fresh mass loss. Sprout analysis was performed at 0, 7, 15, 30, 45, 60, and 90 days of storage. The number of sprouts < 2 mm in length was determined, and the length of sprouts > 3mm was measured with a caliper. Relative sprout incidence was calculated considering the highest number of sprouts set as 100 % (Finger et al., 2018).

*2.4. Carbohydrate analysis*

Fresh flesh samples from tubers after 0, 7, 15, 30, 60, and 90 days of storage were used for carbohydrate quantification. Carbohydrates were extracted from 3 g of samples using 20 mL of 80 % ethanol at 85 °C. Determination of total soluble sugars (TSS) content was performed using the phenol sulfuric acid method (Dubois et al., 1956), and reducing sugars (RS) were determined by the dinitrosalicylic acid method (Gonçalves et al., 2010). The non-reducing sugar (NRS) content was obtained by the difference between the total soluble sugar content and the reducing sugar content. Results were expressed as % TSS, RS and NRS based on fresh matter. The content of alcohol insoluble solids (AIS) was determined by the methodology of Bonte & Picha (2000) and results were expressed as % AIS based on fresh matter.

*2.5. French fries color*

Six tubers were processed into French fries. Potatoes were fried in refined soybean oil for 3 minutes at 180 °C in electric fryers (Ford, Michigan, USA). Scores were subjectively determined by comparing the fry color with the USDA (1967) French fries color chart.

*2.6. Gene expression analysis*

Expression of *KRP1* (Campbell et al., 2012) and *KRP4* (Fischer et al., 2015) genes were determined by quantitative real-time PCR analysis (qRT-PCR). Four tubers from control, DMN, and MeJa treatments were selected before treatment at day 0 and after 7, 15 and 30 days of storage. Samples were rapidly frozen in liquid nitrogen and total RNA was extracted using Trizol reagent (Life Technologies®) as described by the manufacturer. RNA was treated with DNase I (Invitrogen®) and quantified using a Qubit RNA BR kit (Life Technologies®). Additionally, RNA quality was evaluated by 2 % agarose gel electrophoresis and the 260/280 nm and 260/230 nm absorbance ratios. RNA (2 µg) was used for cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems®). Real-time PCR reactions were assembled using the Fast SYBR Green Master Mix System (Applied Biosystems®) and performed on the StepOnePlus™ Real-Time PCR System-Instrument (Applied Biosystems®). The reaction program used an initial denaturation of 20 s at 95 °C, 40 cycles of 3 s at 95 °C and 30 s at 60 °C. The final denaturation of 15 s at 95 °C was used to generate a denaturation curve.

Primers specific for the *KRP1*, *KRP4*, and e*EF-1a* genes (a reference gene) were designed using Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and Oligocalc (http://biotools.nubic.northwestern.edu/OligoCalc.html) with sequences shown in Table 1. A total of 4 biological replicates and 2 technical replicates were performed for each gene. Relative quantification was calculated according to Method $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001), using e*EF-1a* as the internal normalizer.

**Table 1**

Primers were used to determine the expression of the *KRP1* (type 1 cyclin-dependent kinase inhibitor) and *KRP4* (type 4 cyclin-dependent kinase inhibitor) genes.

| **Gen** | **Sequence (5' – 3')** | **Melting temperature** | **Primer efficiency** | **Access code** |
|---|---|---|---|---|
| *KRP* | (F) TCCGGTGGCGAGTTCTGAGA | 62.5 | 95.3 | XM_006361670 |
| | (R) CAACTTCCCCACCGTTGGCA | 62.5 | | |
| *KRP* | (F) TGTTATCTGCAGCTGAGGAGC | 62.1 | 94.2 | NM_001318590 |
| | (R) TCACCCTCAAACTCCGATTCT | 62.1 | | |
| e*EF-* | (F) AGATTGGAAACGGATATGCTCC | 60.9 | 90.3 | AB061263 |
| | (R) TCCTTACCTGAACGCCTGTCA | 61.2 | | |

*2.7. Experimental design and Statistical analysis*

The experiment used a completely randomized design, with split plots. Plots were composed of control, DMN, and MeJa treatments and the subplots were evaluation times. The experiment consisted of three repetitions, containing 2 tubers for each experimental unit. Data were analyzed by analysis of variance using the Statistical Analysis System (SISVAR-UFLA), and means were compared using Tukey's test ($P < 0.05$).

## 3. Results

Loss of fresh weight loss in control tubers was greater than in DMN and MeJa treatments and was significant after 30 days in cold storage (Fig. 1). At 90 days of storage, DMN and MeJa treated potatoes had an average of 53% less fresh weight loss compared to control tubers.

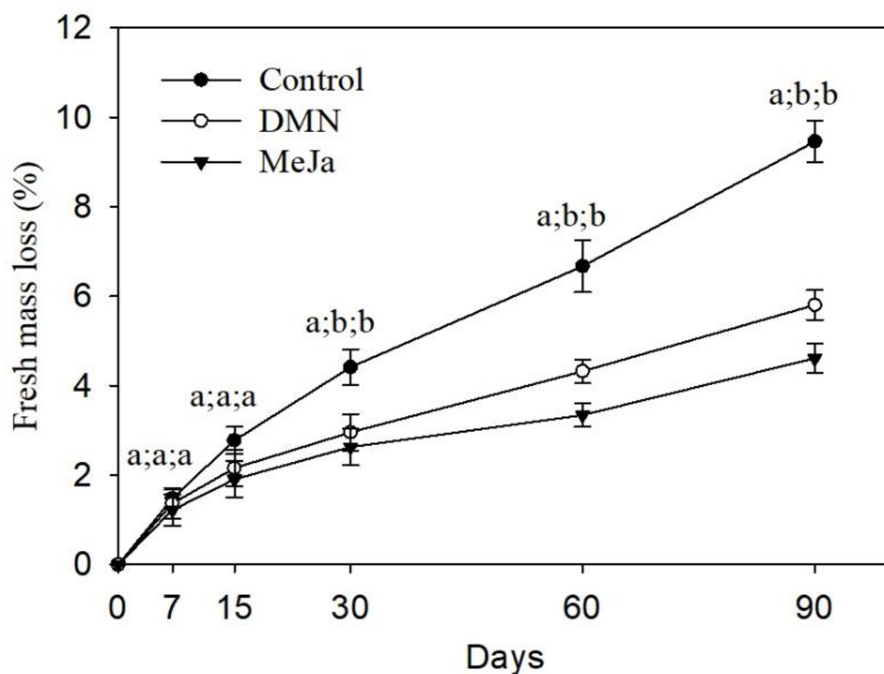


**Fig. 1.** Fresh mass loss (%) in untreated (control) and DMN or MeJa treated potato tubers of cv Challenger during storage at 8 °C. Means followed by the same letter do not differ from each other by Tukey's test at 5 % probability. Vertical bars represent the standard error of the means.

DMN treatment delayed the first visible sign of sprouting beyond 30 days when control and MeJa treated tubers showed first evidence of sprouting (Fig. 2A). There was a significant reduction in the percentage of sprouted buds for DMN and MeJa treated tubers compared to control between 30 and 60 days of storage. In this period, DMN and MeJa reduced the number of measurable sprouts by at least 40 % (Fig. 2A).

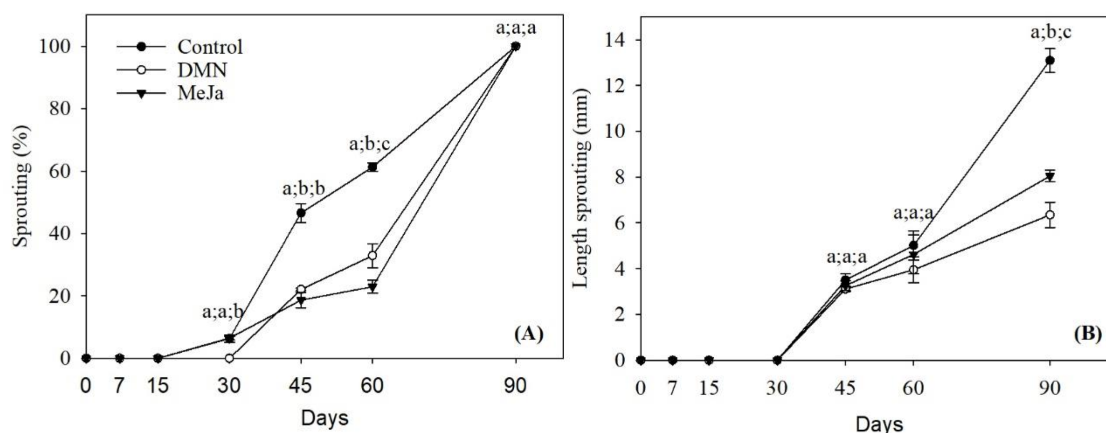


**Fig. 2.** Percentage of sprouting (A) and shoot length (B) in untreated (control), DMN treated, and MeJa treated potato tubers of cv Challenger during storage at 8 °C. Means followed by the same letter do not differ from each other by Tukey's test at 5 % probability. Vertical bars represent the standard error of the means.

Concomitantly to the differences found for the percentage of sprouting among the treatments, there were significant differences in the length of sprouts after 90 days in storage, with DMN being the most effective treatment to reduce shoot length (Fig. 2B). At 90 days, sprouts of tubers treated with DMN, MeJa, or control were an average, 5.1, 6.8, and 13.1 mm in length, respectively (Fig. 2B).

There were significant changes in the soluble carbohydrate content of treated and untreated tubers throughout storage (Table 2). Tubers treated with DMN and MeJa had significantly lower TSS during the last 30 days of storage compared to control tubers. When potatoes in storage are dormant, despite of changes in soluble sugars, starch levels remain almost constant (Lewis et al., 1994). However, a change in respiration such as that which occurs with bud break would probably be linked to sucrose hydrolysis. Therefore, the lower content of

TSS in DMN and MeJa treated tubers was likely due to the lower requirement for sugars as substrates for respiration at 90 days of storage (Table 2).

**Table 2**

Percentage of total soluble sugars (TSS), reducing sugars (RS) and non-reducing sugars (NRS) in untreated (control), DMN treated and MeJa treated potato tubers cv. Challenger during 90 days of storage at 8 °C.

| Days | Total sugars | | | Reducing sugars | | | Non-reducing sugars | | |
|---|---|---|---|---|---|---|---|---|---|
| | C | DMN | MeJ | C | DMN | MeJa | C | DMN | MeJa |
| 0 | 0.26ᵃ | 0.26ᵃ | 0.26ᵃ | 0.05ᵃ | 0.05ᵃ | 0.05ᵃ | 0.21ᵃ | 0.21ᵃ | 0.21ᵃ |
| 7 | 0.31ᵃ | 0.21ᵇ | 0.24ᵃᵇ | 0.04ᵃ | 0.04ᵃ | 0.06ᵃ | 0.27ᵃ | 0.18ᵇ | 0.18ᵇ |
| 15 | 0.30ᵃ | 0.23ᵇ | 0.29ᵃᵇ | 0.10ᵃ | 0.03ᵇ | 0.06ᵃᵇ | 0.20ᵃ | 0.20ᵃ | 0.23ᵃ |
| 30 | 0.30ᵃ | 0.31ᵃ | 0.28ᵃ | 0.07ᵃ | 0.10ᵃ | 0.05ᵃ | 0.23ᵃ | 0.21ᵃ | 0.22ᵃ |
| 60 | 0.27ᵃ | 0.20ᵇ | 0.26ᵃᵇ | 0.10ᵃ | 0.06ᵃ | 0.08ᵃ | 0.17ᵃ | 0.13ᵇ | 0.17ᵃ |
| 90 | 0.36ᵃ | 0.20ᵇ | 0.26ᵇ | 0.22ᵃ | 0.07ᵇ | 0.11ᵇ | 0.13ᵃ | 0.13ᵃ | 0.14ᵃ |

Means followed by the same letter do not differ from each other by Tukey's test at 5 % probability.

To better understand, carbohydrate changes in DMN and MeJa treated tubers, TSS was divided into the RS and NRS. The accumulation of RS significantly varied between treatments, with notable increases for the control at 90 days of storage (Table 2). Control tubers from the beginning of storage to the 90$^{th}$ day increased the RS content by 4.8-fold. In contrast, RS was relatively unchanged in DMN-treated tubers and increased by 1.6-fold in MeJa-treated tubers at 90 days in storage.

Regardless of treatment, NRS decreased during storage (Table 2). However, tubers treated with DMN had a 24 % lower NRS content after 60 days of storage compared to control

and MeJa treated tubers (Table 2). The significant decrease in sucrose content in DMN-treated tubers at 60 days, was not accompanied with an accumulation of RS (Table 2).

Quantifying the content of alcohol insoluble solids (AIS) is an efficient method to measure indirectly starch content in tuberous roots. In the present study, there was no significant variation in AIS content induced by the treatments or by the length of storage, maintaining a general average between 15.0 to 16.7 % (data not shown). At 90 days of storage sprouting just began, especially in potatoes treated with DMN and MeJa.

Color is one of the most important appearance attributes that influence consumer acceptability of processed potatoes and was characterized by frying French fries of control, DMN-treated, and MeJa-treated tubers after a different period of storage. Non-enzymatic browning of French fries was apparent after 7 days of storage (Fig. 3). Treatments with DMN and MeJa maintained acceptable fry color throughout the 90 days of storage. These fries maintained a light golden color according to the scores given by the USDA color chart (Fig. 3).

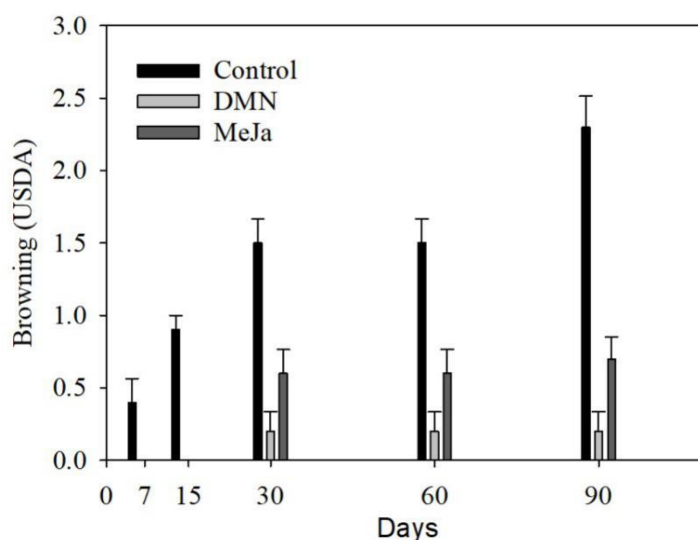


**Fig. 3.** Potato tuber browning scale cv Challenger as a function of storage (days) at 8 °C by comparison with USDA (1967) color scale (0, 1, 2 and 3) for French fries. Bars represent the means ± standard error.

Expression of *KRP1* (type 1 cyclin-dependent kinase inhibitor) and *KRP4* (type 4 cyclin6a-dependent kinase inhibitor) genes in dormant potato tuber tissue was determined on day 0 before the treatment with DMN and MeJa and after 7, 15, and 30 days after the application (Fig. 4). Transcripts changes related to storage duration were detected for both genes by qRT-PCR. Transcripts of the *KRP1* gene of DMN treated tubers also showed a significant increase after 7 days (Fig. 4). Conversely, *KRP4* gene expression levels showed no statistical difference between treatments (Fig. 4).

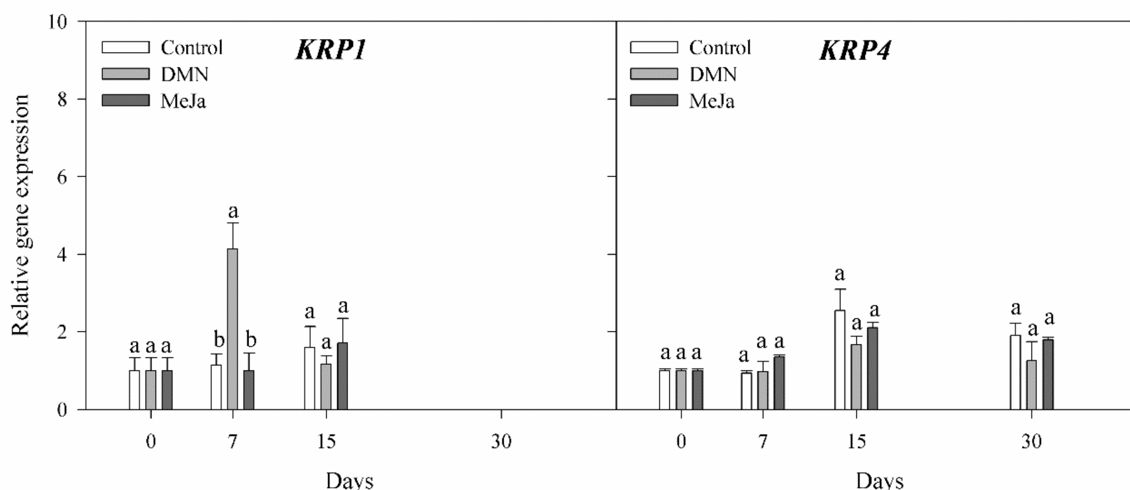


**Fig. 4.** Analysis of *KRP1* and *KRP4* gene expression by qRT-PCR in untreated (control), DMN-treated and MeJa-treated potato of cv Challenger at 0, 7, 15 and 30 days in storage at 8 °C. *KRP1* and *KRP4* transcript levels were normalized using e*EF-1a* as the reference gene and time control 0 was used as the normalizing reference sample. Means followed by the same letter do not differ from each other by Tukey's test at 5 % probability. Vertical bars represent the standard error of the means.

## 4. Discussion

### *4.1. DMN and MeJa application reduced sprouting and fresh mass loss*

The loss of fresh mass in stored potato tubers is an important variable that is directly associated with sprouting and commercial depreciation of the processed products. At dormancy

break, tubers increase the rate that they lose water and dry matter due to an elevation in respiration rate (Tester et al., 2005). The treatments with DMN and MeJa efficiently reduced fresh mass loss, thereby contributing to a reduction of postharvest losses. Similarly, DMN and jasmonic acid have been reported to decrease fresh mass loss in other potato cultivars, including Russet Burbank, Russet Norkotah, Shepody, and Diamant (Kalt et al., 1999; Weerd and Thornton, 2010; Allah et al., 2018).

The increased fresh mass loss in the control tubers can be explained by the increase of axillary bud growth in these tubers initiated 30 days of storage at 8 °C. In contrast, DMN and MeJa treated tubers exhibited less fresh mass loss, possibly due to a reduced respiratory rate in conjunction with sprouting inhibitors maintaining tissue turgor.

The decrease in sprouting incidence, due to the use of alternative products to CIPC such as jasmonates was previously demonstrated by Lulai et al. (1995) and Platonova et al. (2010), which maintained the processing quality of the tubers. Weerd and Thornton (2010) also concluded that the use of naturally occurring volatile compound DMN was effective in reducing sprouting and mass loss in several cultivars of potatoes stored at 7 or 10 °C.

*4.2. Sugar content is affected by DMN and MeJa treatments*

Equally to the results presented lower levels of RS induced by jasmonate treatment was previously reported by Lulai et al. (1995). Allah et al. (2018) also observed that the total sugar content decreased with increasing jasmonic acid dosage in potato cv Diamant. Similarly, to our results, Yang et al. (1999) reported a reduction in reducing sugar content in Russet Burbank tubers when treated with DMN during storage.

Previous studies indicate that decreases in RS in potatoes are due to a lower incidence of sprouting (Jia et al., 2019; Finger et al., 2018; Foukaraki et al., 2016). When the sprouts start to grow, the cell metabolism of the tuber shifts from synthesis to degradation of carbohydrates. In control tubers, there was a higher incidence of sprouting and, consequently, a higher rate of

sucrose hydrolysis increasing the RS content at the end of the storage. The significant decrease in sucrose content in DMN-treated tubers at 60 days, was not accompanied by an accumulation of RS indicating that degraded sucrose in these tubers may be being directed to other metabolic processes, such as resistance mechanisms to various biotic and abiotic stresses (Campbell et al., 2012; Campbell and D'Annibale, 2016).

Starch is the major carbohydrate in potatoes and its concentration is an important index for determining processing quality. Therefore, a large number of hexoses were not needed to support the growth and respiration, remaining AIS above 15 %. It is suggested that the accumulation of RS was due to the degradation of sucrose already existing in the tuber, not due to starch degradation.

*4.3. DMN and MeJa application minimized browning fry color*

French fries from control tubers exhibited a continuous increase of non-enzymatic browning during storage and was already evident after seven days of storage. In this work, we found a close relation between the accumulation of RS and the intensity of non-enzymatic browning in the French fries. Non-enzymatic browning of fry potatoes is due to the Maillard reaction between the free amino group and RS (Mestdagh et al., 2008).

*4.4. DMN and MeJa exhibited differential regulation of KRP1 and KRP4 genes*

Previous work established that DMN does not exert its inhibitory activity by extending the natural period of tuber dormancy (Campbell et al., 2010). Rather, DMN's inhibitory properties in delaying the onset of sprouting are related to disruption of cell cycle progression by blocking the G1/S phase transition by increasing expression of *KRP1* and *KRP4* genes (formerly called *KRP2*) (Campbell et al., 2012), are D-type cyclin inhibitors (CYC-D) that are required for cell cycle continuity (De Veylder et al., 2003; Doonan and Kitsios, 2009). However, Campbell et al. (2012) observations were made only 3 days after the application of

DMN. Our results indicate that the inhibitory effect of DMN, by maintaining *KRP1* gene expression, is more significant than *KRP4* gene expression levels (Fig. 5). However, the decrease in expression of *PRP1* after 15 days from DMN application is noticeable as the storage period increases, which may be related to a loss of inhibitory capacity of DMN. This supports the observed need for multiple applications of DMN as suggested by Knowles et al. (2005).

In contrast, MeJa mode of action in controlling sprouting is not understood, especially at the molecular level. However, the expression levels of the *KRP1* and *KRP4* genes showed no significant difference due to MeJa treatment, suggesting that MeJA does not act as a cell cycle repressor like DMN.

Differences in gene expression for *KRP1* and *KRP4* in DMN-treated tubers may be related to the independence of each gene in controlling cell division. In rice and maize plants, it has been reported that *KRP1* and *KRP4* act independently in blocking the cell cycle, and exhibit inhibitory activity in different cyclin-dependent cell kinases (CDKs) complexes in the cell (Barrôco et al., 2006; Godínnez-Palma et al., 2017). Unlike DMN, MeJa effectiveness in inhibiting sprouting is likely to be linked to other metabolic pathways.

Interestingly, *KRP* family genes are also known to decrease the number of cells and increase cell size when overexpressed in other plant species (De Veylder et al., 2003). DMN may have a similar effect in potato tubers, which result in the shortening of sprout length and reduced fresh weight loss due to increased turgidity of these cells.

## 5. Conclusion

The use of DMN and MeJA, the alternatives sprout inhibitors to CIPC for storage of potato were effective in reducing postharvest losses. DMN and MeJa reduced the incidence of sprouting and fresh weight loss of tubers and reduced accumulation of reducing sugars that promote non-enzymatic browning of French fries. Significant differences in *KRP1* gene expression in DMN-treated tubers, but not MeJa-treated tubers, suggest that DMN, but not

MeJa, suppresses sprouting by blocking cell cycle progression. Conversely, the expression of the KRP4 gene was shown to be unaffected by the treatments.

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## Declaration of Competing Interest

The authors have no conflict of interest to declare.

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# CHAPTER 2

## Induction of dry rot resistance during the storage of potato tubers by using 1,4-dimethylnaphthalene and methyl jasmonate

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**Induction of dry rot resistance during the storage of potato tubers by using 1,4-dimethylnaphthalene and methyl jasmonate**

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**ABSTRACT**

Fourteen species of *Fusarium* have been associated with dry rot occurrence in potato tubers. Losses related to dry rot can reach up to 60% during potatoes storage. One promising way of controlling this disease is by activating natural defense mechanisms via elicitors that induce resistance. Therefore, this study evaluated the effects of 1,4-dimethylnaphthalene and Methyl Jasmonate as resistance inducers to dry rot caused by *Fusarium nirenbergiae* in both resistant (Asterix) and a susceptible cultivar (Challenger) under cold storage. *In vitro* and *in vivo* tests were carried out by evaluating the volume of infection and the activity of antioxidant and defense-related enzymes such as polyphenoloxidase, peroxidase, catalase, ascorbate peroxidase, phenylalanine ammonia-lyase, lipoxygenase, and glucanase along the time of storage. The results indicate that the reduction in the volume of infection as a function of DMN application is not coupled with the expression of evaluated enzymes. Changes observed in such enzymes behavior are likely associated with differences in disease susceptibility between cultivars and not with the applied compounds. Therefore, our data suggest that the efficiency of DMN in reducing the volume of infection is linked to a direct action on the pathogen rather than activation of defense via antioxidant and defense-related enzymes.

**Keywords:** Sprout inhibitors, postharvest diseases, induced resistance, *Solanum tuberosum*

## INTRODUCTION

Potato (*Solanum tuberosum* L.) is the third most important food crop on the planet and one of the most consumed foods in the world - currently showing a constant trade growth in the Brazilian markets (Embrapa 2020). However, to sustain an expanded market, it is quite necessary to establish a regular and continuous supply with suitable raw materials to maintain the production throughout the year.

During long-term storage, potato tubers face a wide range of postharvest diseases that whether not adequately controlled can affect either total yield or product quality (Glazebrook 2005). Injuries to the tuber's peridermis during harvesting and storage may boost the growth of microorganisms, especially those from *Fusarium* species (Nelson et al. 1981).

Fourteen species of *Fusarium* have been associated with dry rot in potato tubers. In Brazil, the most common related species belong to the *F. solani* and *F. oxysporum* complexes. Tubers with symptoms of dry rot are characterized by external darkening caused by necrosis besides dehydration and mycelial growth (Embrapa 2020). Losses related to this disease are about 6 to 25% (Chelkowski 1989; Carnegie et al. 1990), but in severe cases, it can reach up to 60% during storage (Theron 1991).

Currently, the preventive control strategies for dry rot include the use of resistant cultivars and cultural practices, such as the use of healthy seeds and wound healing during the curing process before storage. Resistance is a key element in strategies to control dry rot, however, cultivars present a diversified level of resistance to *Fusarium* spp. Furthermore, none have yet been presented a full resistance to all *Fusarium* species (Corsini and Pavek, 1986). Among the potatoes produced in Brazil concerning industrial processing are the Challenger and Asterix cultivars, which present a higher fitting and yield and good storage capacity (Hzpc, 2021), besides a suitable market acceptance. However, Challenger has been shown a higher degree of susceptibility to dry rot.

Additionally to preventive strategies, healing control can be performed by using chemicals as fungicides. However, due to concerns regarding pathogen resistance to many chemicals and potentially harmful effects on the environment and human health, current production systems require crop protection through both innovative eco-friendly compatible methods and sustainable agriculture rather than only chemical applications (Kuc, 2001; Tian and Chan, 2004).

One way to control dry rot disease is based on the induction of endogenous defense mechanisms via elicitors application. Disease resistance in plants is associated with the activation of a wide range of defense responses that attenuate or hinder the infection at certain stages of pathogen-host interaction. Defense mechanisms include pre-existing inducible biochemical barriers such as enzymes that are subsequently activated by the infection (Vanitha et al., 2009). The induction of resistance against the pathogen often entirely affects cell metabolism, especially the activity of polyphenoloxidase (PPO), peroxidase (POD), catalase (CAT), peroxidase ascorbate (APX), phenylalanine ammonia-lyase (PAL), lipoxygenase

(LOX), and β-1,3 glucanase (GLU) enzymes. However, their behavior in potato tubers submitted to resistance induction via elicitors application remains elusive.

Currently, studies on potato tuber-pathogen interactions have been conducted with regards to *Phytophthora infestans*, *Verticillium dahliae*, and *Rhizoctonia solani* AG3PT (Vleeshouwers et al. 2000; Derksen et al. 2013; Genzel et al. 2018). Nevertheless, studies concerning interactions of potato tubers and *Fusarium* species are scarce, even though potato dry rot is occurring worldwide.

The natural compound 1,4-dimethylnaphthalene (DMN) was isolated from potato tubers and it has been effective in preventing sprout occurrence (Beveridge et al. 1981; Coleman et al. 1981). Although the inhibitory properties related to sprouting growth as well as the commercial utilization of DMN have been well-known and explored over several years, the mechanisms by which DMN exerts other biological effects are poorly understood.

Previous studies have demonstrated reductions in potato qualitative losses after DMN application, which was closely related to a lower incidence of fungal pathogens, thus suggesting a likely fungicidal property (unpublished data). However, it is unclear whether the reduction of disease during storage is a function of the action of DMN directly on the pathogen or a consequence of some endogenous response of the tuber that provides resistance to this disease. Analysis of gene expression in DMN-treated potato tubers showed significant increases in the PR4 and PR5 genes, which are associated with the pathogenic response (Campbell et al. 2016). However, the relationship between DMN fungal pathogen severity has been little investigated.

Jasmonates are a group of well-known compounds that trigger many physiological and biological responses in plants (Wasternack 2007). The exogenous application or biosynthesis stimulation of resistance-inducing organic acids, such as methyl jasmonate (MeJa) - derived from jasmonic acid (JA) - may act as a resistance inducer in plants (Thomma et al. 2000; Belhadj et al. 2000; Belhadj et al. al. 2006; Kepczynska and Krol 2012; Chakraborty et al. 2018). Previous studies have demonstrated a decreased infection of *Rizoctonia solani* AG3PT via systemic transcriptional defense-related genes activation in potatoes, which are closely associated with jasmonic acid signaling pathways (Genzel et al. 2018), highlighting thus its potential as a resistance inducer in an organ-specific manner, namely tubers.

Therefore, this study evaluated the ability of 1,4 - dimethylnaphthalene (DMN) and Methyl Jasmonate (MeJa) in acting as resistance inducers to dry rot caused by *Fusarium nirenbergiae* a *Fusarium oxysporum* complex pathogen, in both resistant and susceptible cultivars during cold storage.

## EXPERIMENTAL METHODS

### *In vitro experiment*

*Fusarium* isolates were obtained via direct isolation from potato tuber tissues with symptoms of dry rot. By proteomic analysis coupled with RT-PCR, the monosporic isolate *Fusarium nirenbergiae* was identified as *Fusarium oxysporum* species complex (FOSC). For the *in vitro* test, mycelium discs (Ø = 6 mm) from five-day-old *Fusarium* culture were sub-cultured into Petri dishes containing PDA (Potato – Dextrose – Agar). These plates were stored in a chamber (3.3 L) containing filter paper soaked by DMN (20 mg $L^{-1}$) and MeJa (10 µM $L^{-1}$) diluted in 2 ml of 95% ethanol. For the control, the plates were also stored in a chamber absent of the compounds, only with 95% ethanol solution.

Each treatment comprised three repetitions. After 1, 2, 4, 6, 8, 10, 14, and 18 d in the chambers, the evaluation of mycelial growth was performed at two diametrically opposite directions using a ruler by considering an average of three readings per replicate. Readings were accomplished when the growth area of the colony covered the diameter of the control plate.

### Bioassay

Asterix and Challenger potato cultivars were grown in the region of Perdizes-MG (19º21'10" S 47º17'34" W), in which all recommended cultural sets of managements were accomplished until the harvest point, totaling a cycle of 120 d. After this timeframe, disease-free and standardized tubers (size between 150-200 g) were cured for seven d at 14 °C and 90% RH.

Subsequently, the tubers were superficially sterilized in 0.5% sodium hypochlorite and rinsed in sterile water before application of the compounds. Afterward, DMN and MeJa were applied via vaporization, according to the method of Vaughn and Spencer (1991), with slight modifications. The tubers were placed in 90 L hermetically sealed containers together with a Petri dish containing volatile compounds. Fumigation treatment was carried out from heating to complete vaporization of 20 mg of DMN and 10 µM MeJa for 1 kg of potato each. After 2 h under a riched atmosphere with either DMN or MeJa, the tubers were transferred to a cold room at 8 °C, under dark, with a relative humidity of 85-90%, for 3 d (time previously determined), to induce defense mechanisms. The control tubers were treated with vaporization of 95% ethanol solution over the same period.

### Tuber inoculation and incubation with *Fusarium nirenbergiae*

After the period of defense mechanisms induction, a uniform wound (5 mm deep and 2 mm diameter) was made at the equatorial region of each tuber using a sterile wooden toothpick, and 20 µL conidial suspension of *Fusarium nirenbergiae* (1 x 106 spores mL). -1) was added to wounded regions. For a more efficient contrast, two control treatments were used from non-treated tubers (without DMN and MeJa) inoculated with distilled water or conidial solution. Subsequently, the tubers were incubated in boxes and covered with plastic film for 48 h to favor the colony growth. Then, the plastic was removed and the boxes remained 22 °C, over the evaluation period. Each treatment consisted of four replications with 20 tubers each, and an experimental unit consisting of two tubers. Peal samples were collected at six different periods, namely 0, 2, 4, 6, 8, and 10 d after inoculation, to verify likely variations between treatments.

**Disease volume**

After the incubation, tubers were cut along the longitudinal axis around the inoculation sites. Disease development was described as the rotten area around each wound. For disease assessment, the width and depth of the symptomatic lesion were measured from each wound site. Based on the method described by Peters et al. (2008), the rot volume of each wound area was calculated using the following equation: Volume = 1/3*π*h*r2, where r is half the rot width, and h is the rot depth.

**The activity of antioxidant system-related enzymes: peroxidase (POD, EC 1.11.1.7), polyphenoloxidase (PPO, E.C. 1.14.18.1), catalase (CAT, EC 1.11.1.6), and ascorbate peroxidase (APX, EC 1.11.1.11)**

The enzymatic extract was prepared by homogenizing 0.2 g of plant material in 2 ml of extraction buffer containing 100 mM potassium phosphate (pH 7.0), 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1% (w/v) of polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 14,000 g for 15 min at 4 °C, in which the supernatants were used to determine CAT, APX, and POD activities.

CAT activity was carried out as described by Havir and McHale (1987). The reaction buffer contained 50 mM potassium phosphate (pH 7.0) and 12.5 mM $H_2O_2$. The reaction was initiated by adding the enzymatic extract followed by readings at 240 nm of absorbance for 1 min. CAT activity was expressed in µmol $s^{-1}$ $kg^{-1}$ protein, considering the extinction coefficient of 36 $\mu M^{-1}$ $m^{-1}$.

APX activity assay was performed according to Nakano and Asada (1981). The reaction medium was composed of 50 mM potassium phosphate (pH 7.8), 0.25 mM ascorbic acid and

0.3 mM $H_2O_2$. The activity was monitored by reducing absorbance to 290 nm for 1 min. The activity was expressed as µmol $s^{-1}$ $kg^{-1}$ protein by using a molar extinction coefficient of 2.8 $\mu M^{-1}$ $m^{-1}$.

POD activity was quantified as previously described by Kar and Mishra (1976). The reaction buffer consisted of 25 mM potassium phosphate (pH 6.5), 20 mM guaiacol and 20 mM $H_2O_2$. The activity was determined by increasing the absorbance to 470 nm and expressed in µmol $s^{-1}$ $kg^{-1}$ protein, by considering the molar extinction coefficient of 26.6 $\mu M^{-1}$ $m^{-1}$.

PPO activity was quantified according to Benjamin and Montgomery (1973) recommendations. Approximately 0.2 g of plant material was homogenized in an extraction buffer containing 0.1 M potassium phosphate (pH 6.5) and 1 mM PMSF. The homogenate was centrifuged at 14,000 g for 15 min at 4 °C. The reaction medium consisted of 0.1 M potassium phosphate (pH 5.0) and 120 mM pyrocatechol. The reaction was started with the addition of enzymatic extracts and the activity was read in a spectrophotometer at 420 nm for 3 min. PPO activity was expressed in µmol $s^{-1}$ $kg^{-1}$ protein, by using a molar extinction coefficient of 3.45 $mM^{-1}$ $m^{-1}$.

The protein concentration in each sample was determined according to the colorimetric method described by Bradford (1976).

**The activity of defense system-related enzymes: β-1,3-glucanase (GLU, EC 3.2.1.39), phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), and lipoxygenase (LOX, EC 1.13.11.12)**

GLU activity was accomplished in an enzymatic extract obtained from homogenized 0.2 g of plant material in 2 mL of 0.1 M potassium phosphate buffer (pH 6.8) containing 1 mM of phenylmethylsulfonic fluoride (PMSF) and 0 0.1 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 20,000 × g for 25 min at 4 °C. Enzyme activity was determined as described by Miller (1959), with minor modifications. The reaction was started by adding 100 mM sodium acetate buffer (pH 5.0) and laminarin substrate at a concentration of 4 mg/mL. The reaction mixture was incubated in a water bath at 45 °C for 45 min. After the incubation timeframe, the content of reducing sugars was assessed by adding dinitrosalicylic acid to the mix, followed by incubation in a water bath for 5 min at 100 °C. The reaction was inactivated by cooling the samples in an ice bath. The absorbance of the GLU released product was measured at 540 nm, with the activity being expressed as µmol $s^{-1}$ $kg^{-1}$ protein.

Phenylalanine ammonia-lyase (PAL) was quantified according to Ke and Saltveit (1986), with modifications, via the conversion of L-phenylalanine to cinnamic acid. The enzymatic extract was obtained from approximately 0.2 g of plant material homogenized in an extraction buffer composed of borate buffer (100 mM/pH 8.5) and 10 mM of 2-mercaptoethanol. The homogenates were centrifuged at 20,000 x g for 20 min at 4 ºC. The activity was assessed by using an extract consisted of borate buffer (pH 8.5) and 100 mM L-phenylalanine. Then it was incubated at 40 °C for 60 min. The reaction was inactivated by the addition of 5 N HCL before readings at 290 nm. The samples were twice centrifuged at 10,000 x g for 5 min before further reading. The phenylalanine ammonia-lyase activity was expressed in µmol $s^{-1}$ $kg^{-1}$ protein.

For enzymatic determination of LOX, 0.2 g of plant material was homogenized in an extraction buffer composed of 20 mM sodium phosphate buffer (pH 6.8) containing 1% Triton-X (v/v) and 1% PVP. The homogenate was centrifuged at 15,000 × g for 10 min at 4 °C. The reaction started after adding a mixture containing 50 mM sodium phosphate buffer (pH 6.5) and 10 mM sodium linoleate substrate to the extract. LOX activity was determined according to Axelrod et al. (1981) method. The absorbance of the LOX released product was measured in a spectrophotometer at 234 nm. A molar extinction coefficient of 25,000 $M^{-1}$ $cm^{-1}$ was used to determine the activity of LOX, which was expressed as $Ms^{-1}$ $mg^{-1}$ protein.

The protein concentration in each sample was determined according to the colorimetric method postulated by Bradford (1976).

**Experimental design and statistical analysis of data**

The *in vivo* experiment was conducted in a completely randomized design, in a split-plot arrangement, with the plots as DMN and MeJa treatments, and subplots as the six evaluated periods. For both experiments, data were analyzed by analysis of variance with the Statistical Analysis System (SISVAR-UFLA). If ANOVA showed significant effects, the differences between the means were examined by the Tukey test.

**RESULTS**

***Effect of DMN and MeJa on Fusarium nirenbergiae mycelial in vitro growth***

Colonies of *Fusarium nirenbergiae* isolate exhibited reduced mycelial growth under DMN conditions as compared to control and MeJa for up to eighteen days after inoculation (Figure 1). The average size of the *F. nirenbergiae* colony owas 59 mm for both, while exposure

to DMN resulted in a reduction in mycelial growth around 10 mm up to 18 d. The difference was statistically significant from 24 h after contact with naphthalene. On the other hand, in the presence of jasmonate, mycelial growth was inferior to the control only from 2 to 6 d of incubation, with an increased growth after this period. Treatment with MeJa also demonstrated a lower efficiency as compared to DMN (Figures 1 and 2).

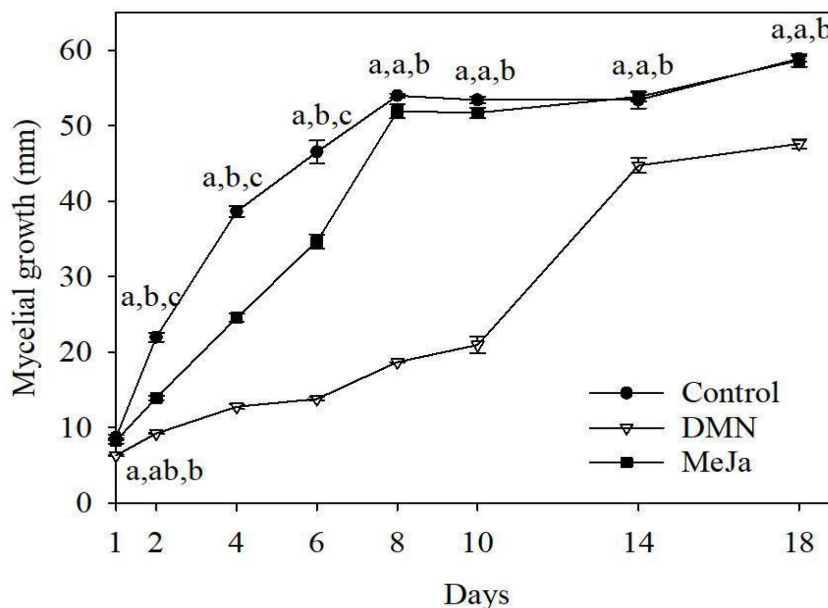


**Figure 1.** Effects of DMN and MeJa treatments on mycelial growth (mm) of *Fusarium nirenbergiae* after the incubation at 22 °C during 18 d. Means followed by the same letter do not differ by Tukey test at 5% probability. Vertical bars represent the standard error of the means.

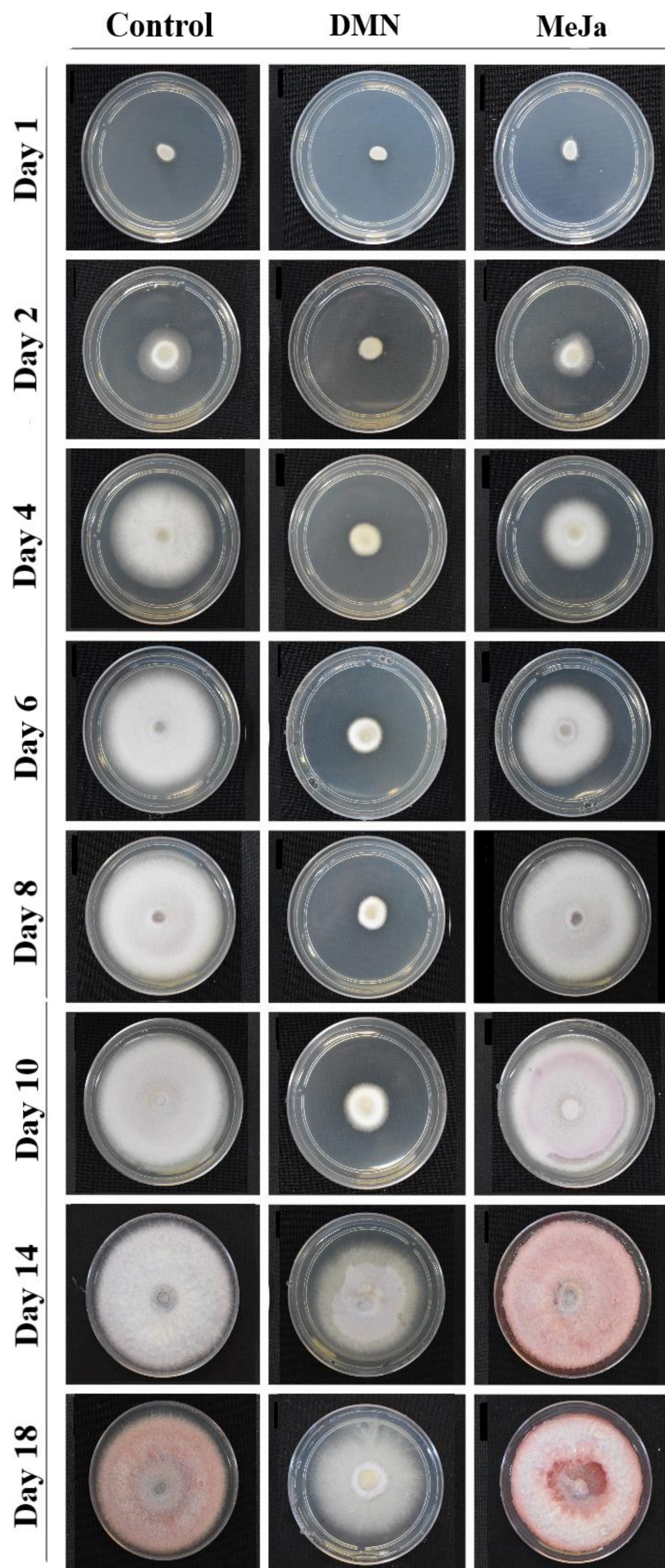


**Figure 2.** Visual analysis of the effect of DMN and MeJa treatments on the mycelial growth of *Fusarium nirenbergiae* after the incubation at 22 °C during 18 d.

***Effect of DMN and MeJa on lesion volume in both Asterix and Challenger potato tubers inoculated with Fusarium nirenbergiae***

The data observed in Figure 3 demonstrate a statistical difference regarding disease infection volume between treatments during the incubation period when both cultivars were compared. The manifestation of the disease started on the 16$^{th}$ d after inoculation in both cultivars. However, Asterix showed a lower disease severity as compared to Challenger. In Asterix, DMN treatment differed from both control and MeJa treatments at 15 d after initial disease symptom occurrence. On 20$^{th}$ d, the DMN treatment presented an efficiency of 25% and 50% in controlling dry rot as compared to control and jasmonate-treated tubers, respectively (Figure 3).

The efficiency of naphthalene was also observed in Challenger tubers from 10 d after treatments application when compared to MeJa. At 15 and 20$^{th}$ d, DMN showed an effectiveness of 75% on average in reducing disease volume, as compared to the control, controlling up to five times the disease at the end of the treatment. Otherwise, MeJa, at 15$^{th}$ d of incubation, displayed a higher disease severity as compared to both DMN and control, in a manner that the dry rot intensified twice as much until the end of the evaluation period (Figure 3).

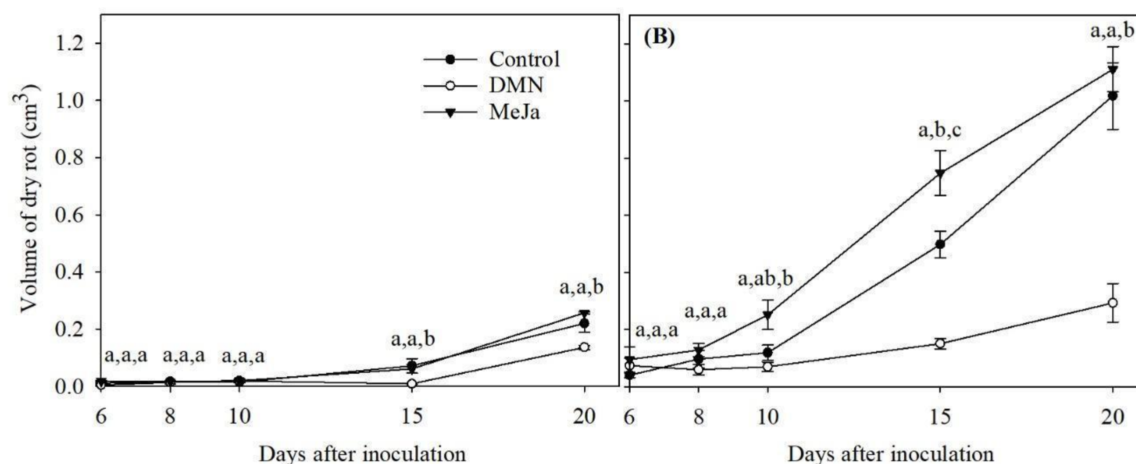


**Figure 3.** Effects of DMN and MeJa treatments on dry rot development in Asterix (A) and Challenger (B) potato tubers inoculated with *Fusarium nirenbergiae* after the incubation at 22 °C during 20 d. Means followed by the same letter do not differ by Tukey test at 5% probability. Vertical bars represent the standard error of the means.

An illustration of dry rot development is observed by a representative tuber at each time of evaluation (Figures 4 and 5). In Asterix, the infection was less intense than in Challenger in

both treatments (Figure 4). Overall, a considerable increase in disease volume is observed in Challenger MeJa-treated tubers at the end of the incubation period as compared to control (Figure 5).

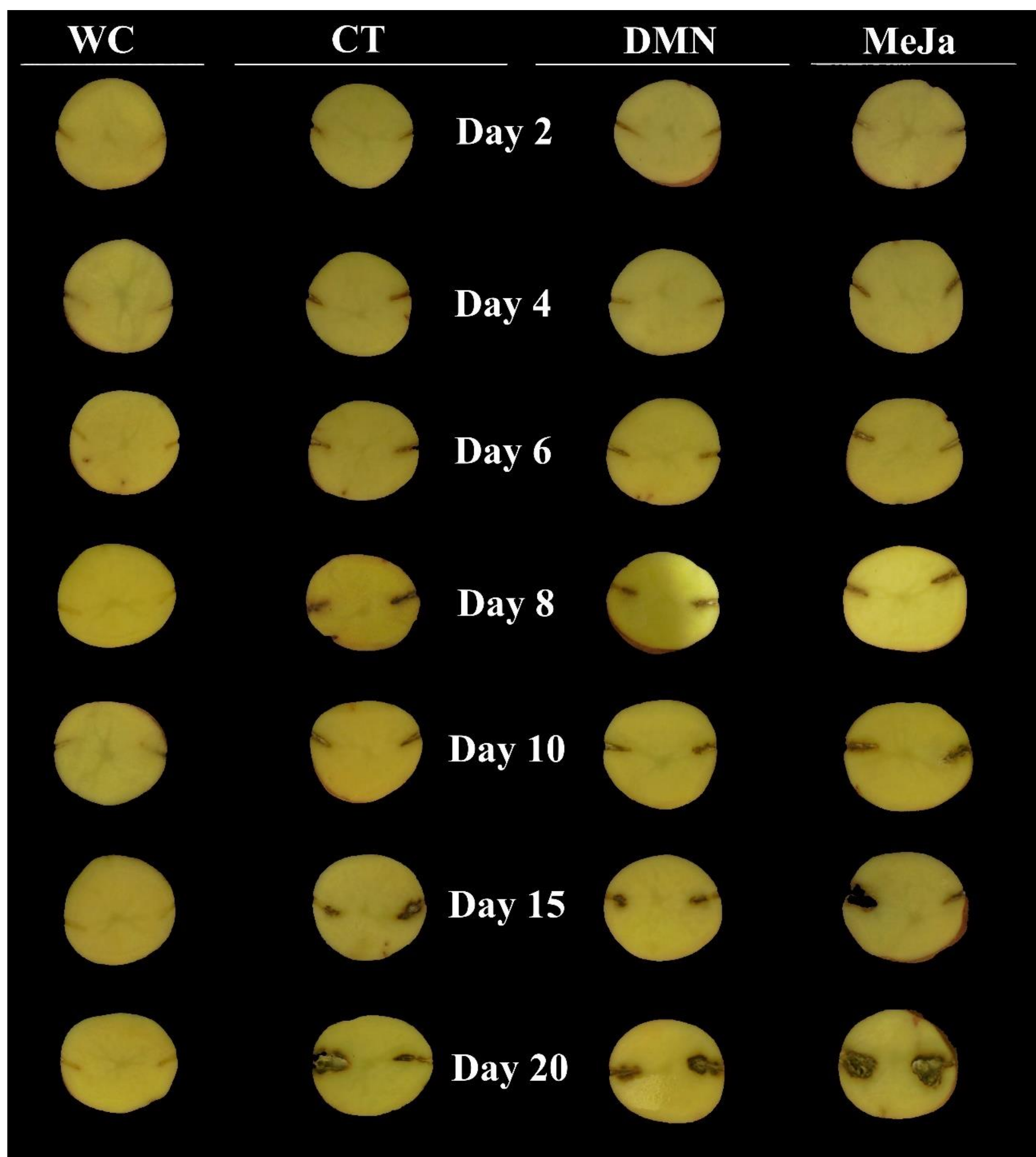


**Figure 4.** Visual analysis of the effect of DMN and MeJa treatments on dry rot development in Asterix potato tubers inoculated with *Fusarium nirenbergiae* after the incubation at 22 °C during 20 d. WC (Tubers inoculated with distilled water); CT (Control tubers inoculated with *F. nirenbergiae*).

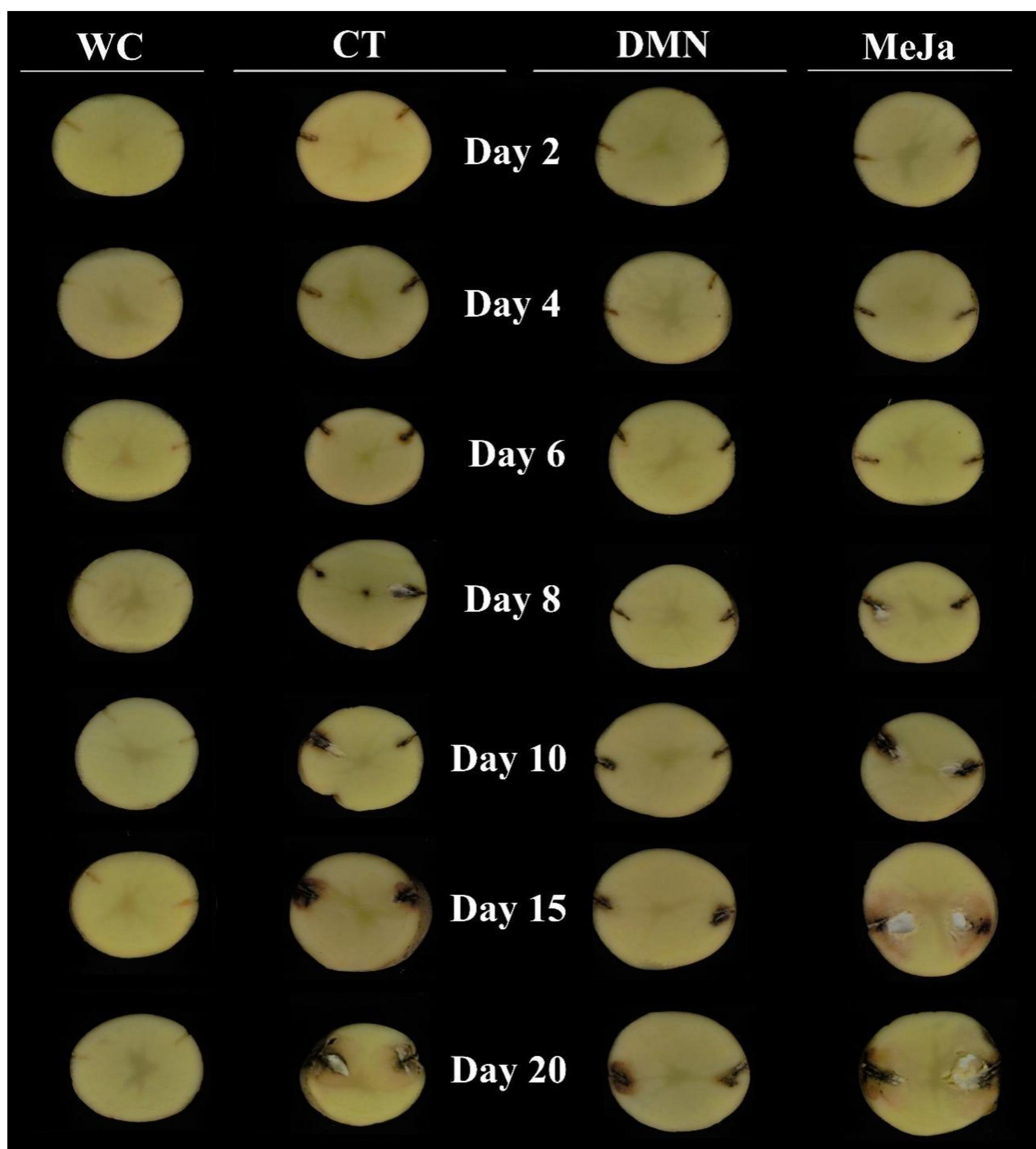


**Figure 5.** Visual analysis of the effect of DMN and MeJa treatments on dry rot development in Challenger potato tubers inoculated with *Fusarium nirenbergiae* after the incubation at 22 °C during 20 d. WC (Tubers inoculated with distilled water); CT (Control tubers inoculated with F. nirenbergiae).

***Effect of DMN and MeJa on the activity of PPO, POD, CAT and APX enzymes***

The data presented in Figure 6 indicate differences in polyphenoloxidase (PPO) activities, and also in peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) enzymes throughout the time. In Asterix tubers, PPO activity differed significantly between treatments at $10^{th}$ d, in a manner that DMN and MeJa led to increased activity as compared to controls (Figure 6 A). In Challenger, significant differences between treatments containing

inoculated *F. nirenbergiae* were observed at 4 and 10 d after treatments application. MeJa-treated tubers showed higher enzymatic activity, and shortly after by treatment with DMN and controls.

Unlike PPO, POD activity was markedly superior in Asterix tubers (Figure 6C). In Challenger, POD did not show a significant difference between treatments containing *F. nirenbergiae* (Figure 6 D). Likewise, CAT activity as a function of the time was significant only in Asterix after inoculation (Figure 7 E). Finally, CAT activity between treatments was evidenced only on the eighth day of incubation. The DMN and MeJa-treated tubers showed a considerable increase in activity, however, at the end of the period of evaluation, there was a significant increase in control; 3x greater as compared to the two treatments, besides a marked reduction in CAT activity in DMN-treated tubers.

APX activity differed between cultivars, with a higher activity in Asterix as compared to Challenger (Figure 7 G and H). Regarding treatments, DMN-treated tubers showed a higher expression among treatments containing inoculated *F. nirenbergiae*, in both cultivars at 10 d after inoculation.

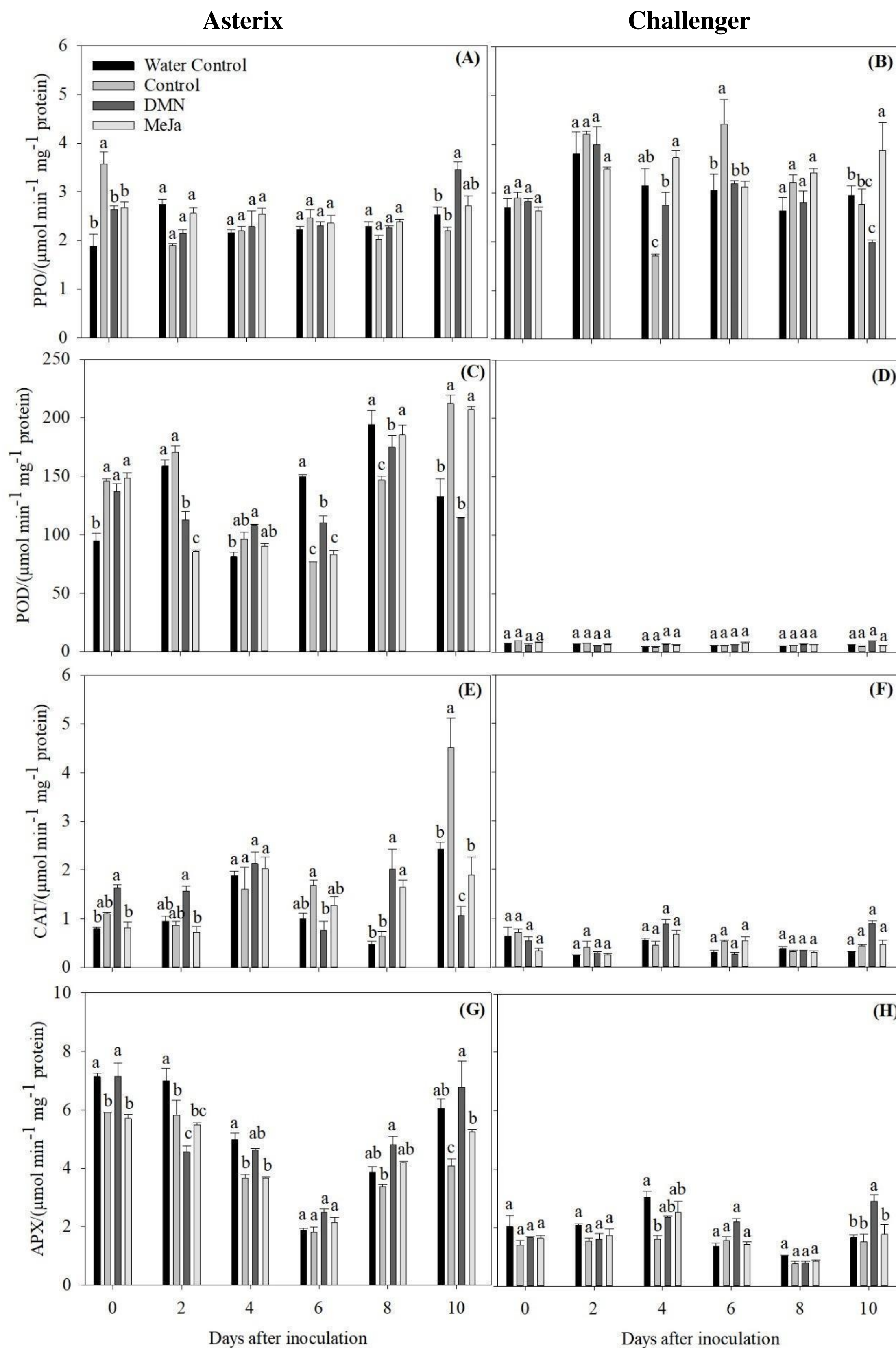


**Figure 6.** Effects of DMN and MeJa treatments on the activity of Polyphenoloxidase (PPO - A and B), Peroxidase (POD - C and D), Catalase (CAT - E and F) and Ascorbate Peroxidase (APX - G and H) enzymes ) in both Asterix and Challenger potato tubers inoculated with Fusarium

nirenbergiae after the incubation at 22 °C over 10 d. Means followed by the same letter do not differ by Tukey test at 5% probability. Vertical bars represent the standard error of the means.

***Effect of treatment with DMN and MeJa on the activity of enzymes PAl, LOX and GLU***

Figure 7 shows differences in defense system-related enzymes activity, such as phenylalanine ammonia-lyase (PAL), lipoxygenase (LOX) and β-1,3-glucanase (GLU) between Asterix and Challenger cultivars throughout the time. PAL activity was not remarkable in Asterix tubers, thus demonstrating significant differences between treatments only for Challenger cultivar (Figure 7 A and B). DMN significantly increased PAL activity by around 25% at each period evaluated, as compared to treatments containing inoculated *F. nirenbergiae*. However, at the end of the experimental period, MeJa also led to a significant increase in it, as the same for DMN (Figure 7A).

The LOX activity showed a significant difference between treatments for both cultivars. Meja-treated tubers showed a significant increase in LOX activity on the $18^{th}$ d as compared to their counterparts. In contrast, DMN reduced LOX activity at 10 d after inoculation (Figure 7C). In Challenger, DMN and MeJa-treated tubers did not exhibit a higher enzymatic activity as compared to controls over the incubation period (Figure 7 D).

Finally, treatments did not induce a significant increase in GLU activity in both cultivars (Figure 7 E and F).

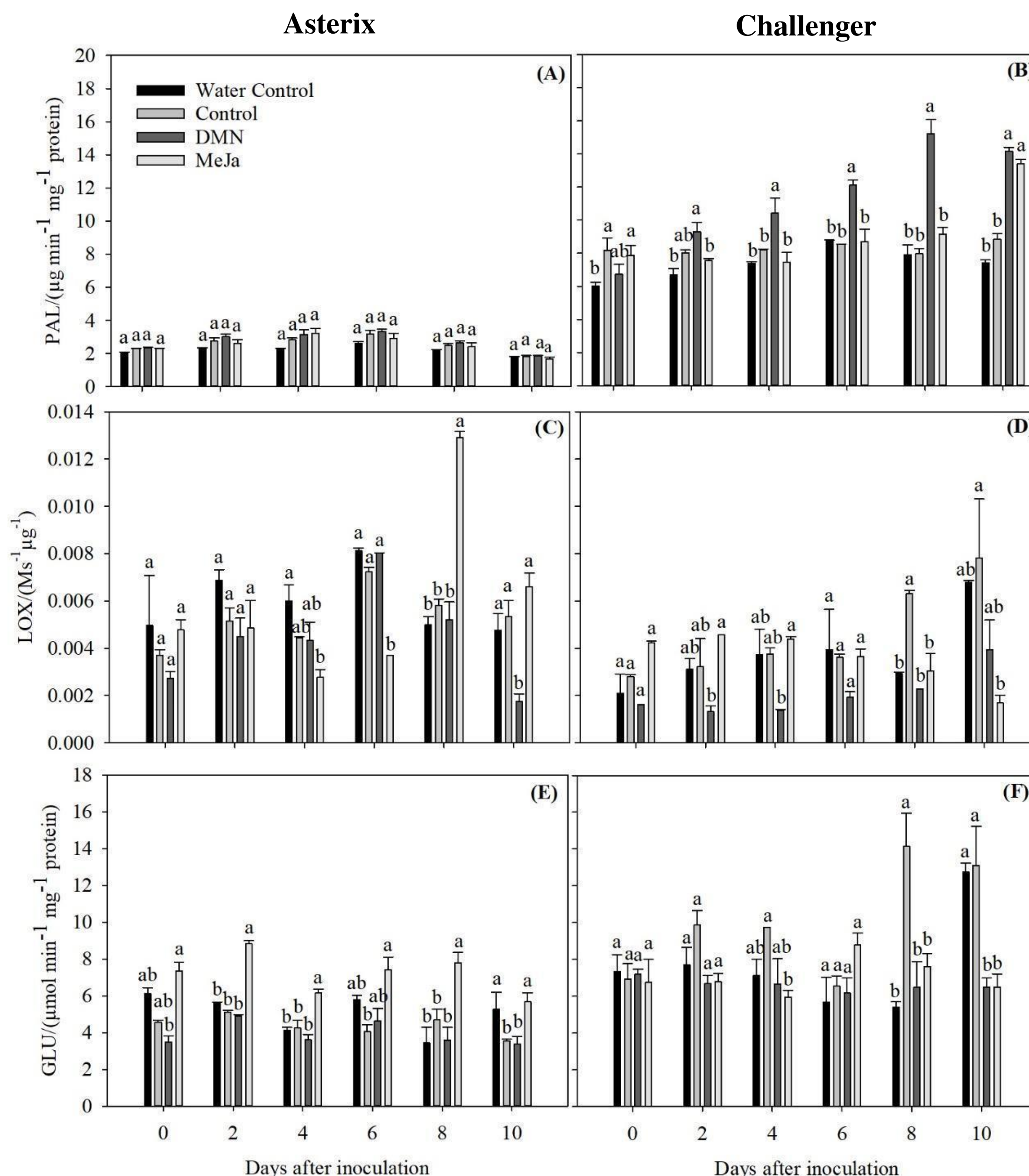


**Figure 7.** Effects of DMN and MeJa treatments on the activity of phenylalanine ammonia-lyase (PAL), lipoxygenase (LOX) and β-1,3-glucanase (GLU) enzymes in Asterix and Challenger potato tubers inoculated with *Fusarium nirenbergiae* after the incubation at 22 °C over 10 d. Means followed by the same letter do not differ by Tukey test at 5% probability. Vertical bars represent the standard error of the means.

## DISCUSSION

*Fusarium* is one of the most severe pathogens affecting stored potatoes worldwide, thereby causing dry rot diseases. The lack of alternative methods to control such severity has led to significant losses during the storage of potatoes. Our study demonstrated that 1,4 - dimethylnaphthalene (DMN) and methyl jasmonate (MeJa) were efficient in inhibiting the

mycelial growth of *Fusarium nirenbergiae* growing under *in vitro* conditions as compared to the control (Figure 1 and 2). However, DMN proved to be more efficient than MeJa until the end of the evaluated period by reducing colony growth up to twice as much as compared to MeJa at 10 d after the incubation beginning. However, both treatments did not inhibit sporulation or kill the fungus, but reduced mycelial growth, demonstrating fungistatic and non-fungicide properties at the applicated concentrations used to control sprouting in potato tubers.

This result is in agreement with previously observed by Campbell et al. (2019), which evidenced a fungistatic activity of DMN on Ascomycota and Oomycota species, also using the recommended dose to control potato sprouting. Therefore, it is suggested that the suppression of fungal growth by DMN seems to be a broad-spectrum response and does not involve the inhibition of specific pathways of the studied species. Likewise, confirming such results, Droby et al. (1999) and Yao and Tian (2005) observed insufficient inhibitory effect of MeJa and Jasmonic Acid (JA) on mycelial growth and spore's germination of *Penicillium digitatum* and *Monilinia fructicola*, as compared to the control under *in vitro* conditions.

In close agreement with what was observed by *in vitro* data, our results showed that the DMN postharvest treatment markedly delayed dry rot lesion volume in potato tubers inoculated with *F. nirenbergiae*. Furthermore, they revealed that there are significant differences regarding susceptibility between cultivars against *F. nirenbergiae* infection. Cultivar Asterix displayed a reduced volume of infection, whereas Challenger presented a higher one (Figures 3, 4 and 5). Based on the Potato Variety Database (AHDB), Asterix is classified as a *F. coeruleum* resistant cultivar (8) and moderately resistant (4) to *F. sambucinum*. In other databases, information on resistance to dry rot is not present or superficially presented, besides not related to individual species. Thus, the interaction of cultivar and *Fusarium* species indicates that resistance to a *Fusarium* spp. does not imply the same level of resistance to all *Fusarium* spp. On the other hand, there is a lack of information regarding resistance to dry rot in the Challenger cultivar, positively corroborating with its susceptibility observed in the present study.

MeJa treatment has been shown to be inefficient in inhibiting disease spread at long-term exposure, as compared to DMN under both *in vitro* and *in vivo* conditions, thereby presenting a higher volume of infection than DMN and the Challenger control. The low efficacy of MeJa was also observed by Yao and Tian (2005), which demonstrated that the product did not significantly reduce the incidence of brown rot in cherry fruits, as compared to the control.

However, previous studies have also indicated that MeJa can directly influence several fungal pathogens. Treatment with MeJa was effective in reducing *Penicillium digitatum* infection in Grapefruit fruits (Droby et al., 1999). Likewise, Fugate et al. (2012) observed that

MeJa reduced the evolution of rot symptoms caused by *Botrytis cinerea*, *Penicillium claviforme* and *Phoma betae* in sugar beet. Therefore, such divergences in results may be associated with the range of species sensitivity to MeJa, and also with the time of exposure, concentration, organ development stage, and way of compound application.

Plant protection against the colonization of fungal pathogens occurs mainly through the activation of a highly coordinated structural and biochemical defense system that prevents the spread of disease. The role of the PPO enzyme in disease resistance has been postulated by many authors (Kumar et al., 1999; Li and Steffens, 2002; Lozovaya et al., 2006). This enzyme participates in the oxidation of phenolic compounds, leading to the production of quinones, which are toxic to several pathogens (Campbell and Sederoff, 1996). In this study, the PPO enzymatic activity did not differ between cultivars. A significant response, however, was observed among treatments only in the Challenger at the end of the evaluation period. Nevertheless, even though it was effective in reducing infection in Asterix and Challenger, DMN did not induce PPO expression (Figure 6 A and 6 B).

Peroxidases are associated with the antioxidative defense system and also lignin biosynthesis process that reinforces the cell wall, impeding the action of lytic enzymes produced by pathogens (Kvaratskhelia et al., 1997). Interestingly, both cultivars differed markedly in POD activity. Asterix potato tubers showed a higher enzyme activity, suggesting that POD is closely related to the degree of cultivar resistance and not to the DMN effectiveness. Naphthalene reduced the infection volume; however, it was not efficient to increase the enzyme activity as compared to other treatments, thereby reducing it at the end of the incubation period.

Both CAT and APX are also reactive oxygen species (ROS) scavenging system-related enzymes. They are either absent or present at low levels before infection, being activated in response to the pathogen presence or produced from a remote precursor. In the same way as POD, CAT did not show increased activity in Challenger potato tubers. APX, additionally to present low levels of activity compared to Asterix, the treatments also did not affect its activity.

It is suggested that the low activity of POD, CAT, and APX enzymes in Challenger tubers may be associated with the cultivar's susceptibility to dry rot. Asterix showed higher activity in the antioxidant system-related enzymes, which is supported by the lower volume of infection by *F. nirenbergiae* (Figure 3 and 4), as compared to Challenger (Figure 5). We suggest that shortly after the inoculation, there was a higher activation of genes related to the initial mechanisms involved with the signal perception and transduction, which induces subsequently the synthesis of defense compounds in both treatments. Thus, the effectiveness of DMN in controlling the infection is not related to direct increases in the expression of such enzymes.

Previous studies indicate that MeJa acts as a resistance inducer by stimulating the production of defense compounds, such as terpenoids, besides activating the expression of enzyme related genes (Thaler, 1999). Despite this, the present data showed that MeJa did not increase the activity of ROS scavenging system-related enzymes. The increase in both defense-related enzymes activity besides the increased volume of infection in Challenger's tubers may indicate a likely hypersensitivity response (Zimmerli et al., 2000). We believe that as an alternative to delaying the infection, the hypersensitivity response was induced in the tuber, which is a common response widely observed in other parts of the plant. Since *Fusarium* is a necrotrophic fungus - hold the ability to extract nutrients from dead host tissues - it provided the fastest expansion of the infection.

The activation of defense-related enzymes occurs through pre-formed biochemical factors in the plant. The degree of biochemical factors involved in plant resistance responses varies according to the pathosystem or by the affected organ and/or tissue, nutritional status, and environmental conditions within the same pathosystem (Johal et al., 1995). The reaction to infection may vary as to how the pathogen is colonized. Necrotrophic microorganisms such as *Fusarium* act by suppressing these mechanisms. Therefore, strategies can be developed against the pathogen, such as increasing the expression of defense-related enzymes such as PAL, LOX, and GLU.

PAL is an enzyme from the secondary metabolism and is closely related to plant resistance to pathogens. Notably, it is involved in the synthesis of phenylpropanoids, such as phytoalexins and lignin, which lead to a higher resistance to pathogens by cell walls reinforcement (Nakazawa et al. 2001). In contrast to the activity of antioxidant system-related enzymes, PAL activity was higher in Challenger potato tubers (Figure 7B). DMN significantly increased PAL activity over the incubation period, indicating that such increased activity may be associated with the volume of infection reduction.

Likewise, LOX and GLU are believed to contribute to the generation of elicitors that hold the potential to activate defense responses, such as cell wall degradation of fungal pathogens (Mohammadi et al., 2002; Axelrod et al., 1981). Cultivars did not differ significantly regarding the activity of both enzymes. Likewise, the treatments did not affect the activity of such enzymes during the evaluated period. Unlike the observed responses in other here studied enzymes, MeJa increased the activity of LOX and GLU enzymes at the end of the incubation period, as compared to its counterpart treatments (Figure 7). In close agreement with this, MeJa increased GLU activity in cherry fruits, although this induced defense response had not reduced the incidence of brown rot caused by *M. fructicola* (Yao and Tian, 2005).

The present data indicate that the reduction in infection severity as a function of DMN application is not directly related to the activity of antioxidant system-related enzymes. Likewise, marked alterations in PAL are also not enough evidence to affirm that DMN actively induces the defense system. We believe thus that changes in such enzymes are probably associated with differences in disease susceptibility between cultivars rather than with the applied compounds. Hence, the results generate a new hypothesis to be further addressed: would the efficiency of DMN in reducing the volume of infection be directly linked to the fungistatic activity rather than the activation of the tuber defense system?

## CONCLUSION

These findings suggest that DMN has shown to be a useful and promising alternative compound in delaying dry rot infection caused by *Fusarium nirenbergiae*. However, further studies must be carried out to understand which mechanism is involved, or whether its effect is directly related to the pathogenicity ability without inducing a plant organ defense system. Finally, significant changes in the activity of enzymes POD, CAT, and APX between cultivars evidenced the natural level of resistance present in both Asterix and Challenger cultivars.

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