Understanding the effect of postharvest tomato temperatures on two toxigenic *Alternaria* spp. strains: growth, mycotoxins and a cell wall integrity-related gene expression

Running title: Effect of postharvest tomato temperatures on toxigenic *Alternaria*

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ABSTRACT

Background

Tomato fruits are susceptible to *Alternaria* spp. spoilage. A correct postharvest management is necessary to prevent mould growth and mycotoxin accumulation, being the temperature one of the main factors. The effect of different postharvest temperatures (5, 12, 25 and 35 °C) on growth, mycotoxin production and a stress-related gene expression by two *Alternaria* spp. was assessed.

Results

Growth rates decreased rapidly when temperature was higher than the optimum (25 °C), while a gradual reduction was detected at lower temperatures. Tenuazonic acid (TeA) was strongly synthesised at all temperatures evaluated, with a maximum between 12 and 25 °C. Alternariol monomethyl ether (AME) was produced only at the two lowest temperatures; with a peak at 12 °C. Regarding the expression of the stress-related *RHO1* gene, during active fungal growth both *Alternaria* spp. showed more copies of the gene as temperature increased. At the stationary phase, the *RHO1* gene expression was significantly higher at 12 °C, coinciding with AME highest accumulation.

Conclusion

Changes on temperatures related to different postharvest stages of tomato fruits markedly affect toxigenic *Alternaria* spp. The highest levels of both mycotoxins were recorded at 12 °C, a common storage temperature for tomato fruit. Additionally, an association between alternariols biosynthesis and the cell wall integrity pathway was noticed in relation to temperature, suggesting that temperature may act as stressor stimulating the *RHO1* gene expression, which in turn triggers this mycotoxin synthesis. These results will be useful in

developing new strategies to efficiently control *Alternaria* spoilage in tomato fruit and by-products.

Keywords: Food safety; tomato fruit; *Alternaria;* tenuazonic acid; alternariol monomethyl ether

1. Introduction

Tomato (*Solanum lycopersicum*) is an important vegetable crop worldwide, both for fresh consumption and industrialisation. In Argentina, it is one of the main horticultural products in the fresh market, with an average yield of 670,000 tons/year.[1] In addition, its production is continuous during the whole year due to the extension in latitude of the crop area throughout the country. Tomato fruits are highly susceptible to fungal contamination both with phytopathogenic species infecting in the field and saprophytes at postharvest stage, which results in economic losses for producers. Due to their thin skin, fruits are highly vulnerable to the disease known as blackmould, produced by small-spored *Alternaria* spp. It is characterised by circular to irregular, slightly sunken, dark green to black patches wounds, and affects the fruits mainly after harvest.[2,3] Thus, an effective control of the storage of tomato fruits is a crucial step to prevent undesirable fungal development. Temperature is one of the main factors involved in spoilage, considering that tomato fruits are exposed to a wide range of temperatures from harvest to consumers. Harvest is performed in summer, when temperatures in the field are above 30 ºC. Later, the fruits can remain at room temperature until they are sent to refrigerated cameras, where temperature drops under 15 ºC. Retailing is usually performed at room temperature, while household storage is usually at temperatures <7 ºC.

After growing under propitious environmental conditions, small-spored *Alternaria* spp. may produce a wide variety of secondary metabolites, including mycotoxins. Among them, alternariol (AOH), its derivative alternariol monomethyl ether (AME), altenuene (ALT), tenuazonic acid (TeA), tentoxin (TEN) and altertoxins I, II, III (ATX-I, -II, -III) are considered the main *Alternaria* mycotoxins.[4,5] TeA has been reported to be acutely toxic for

several animals such as mice, chickens and dogs, and associated with human haematological disorders like Onyalai.[6-9] AOH and AME are mutagenic and genotoxic in bacterial and mammalian cells *in vitro*.[5,10] The presence in food of these two toxins has been associated with high levels of human oesophageal cancer in China.[8,11] Even though there are still no international regulations for any of the *Alternaria* mycotoxins in food and feed, the European Food Safety Authority (EFSA) recently highlighted that the major contribution of *Alternaria* spp. toxins to the diet is due to tomato and tomato-based products consumption.[12,13]

Both growth and mycotoxin production are affected by environmental and nutritional factors.[14] Fungi have different intracellular pathways that allow them to cope with unfavourable external conditions. Among them, the cell wall integrity pathway (CWI) is responsible for the maintenance of the cell wall structure. When detecting cell wall stress, it responds activating its biosynthesis, actin organization and other events.[15] Rho1 is a small G protein coding for the *RHO1* gene which is considered the master regulator of CWI signalling.[16] Changes in the environment constituting a stressful situation for the fungus may trigger modifications at the transcriptional level, which could be related to the regulation of mycotoxin biosynthesis.[17-22] Therefore, it is important to comprehend modifications in these intracellular pathways in relation to mycotoxin accumulation in food. In order to develop control strategies to avoid *Alternaria* spp. contamination in tomato fruits, it is necessary to understand how environmental conditions at postharvest stage can modulate *Alternaria* spp. physiological behaviour, including intracellular response pathways, which might be related to mycotoxin biosynthesis. Thus, the aims of the present study were to: a) evaluate the effect of different postharvest temperatures on the expression

of the *RHO1* gene, a key gene from the CWI in *Alternaria* spp.; and b) correlate such gene expression with their growth and mycotoxin production on a tomato-based medium.

2. Materials and Methods

2.1. Fungal strains

Two small-spored *Alternaria* strains isolated from Argentinean tomato fruits were used in this study. Morphological classification at species-group level according to Simmons[23] and determination of their mycotoxigenic potential *in vitro* had been performed in a previous study.[24] One of the strains, belonged to *A. arborescens* sp.-grp. (Id. T_2_L), being a TeA producer, while the other was classified as *A. tenuissima* sp.-grp. and produced AME (Id. T_50_H). Both strains are maintained in the Food Mycology Group Culture Collection from the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires.

2.2. Culture medium

In order to simulate tomato fruit nutrients, Tomato Pulp Agar (TPA) medium was prepared as described in Vaquera *et al*.[25] Briefly, whole fresh fungicide-free washed tomatoes were blended in a food processor Thermo Mix™, including the peel and the pulp. A portion of 800 mL of this paste was diluted with 200 mL of distilled water and 15 g bacteriological agar (Scharlab S.L., Spain) were added. After sterilization, pH was measured with a BASIC 20 from Crison Instruments S.A. (Barcelona, Spain) and was of 4.34. Also, the water activity level (a_w) was measured by a Novasina Lab Master from Novasina AG (Switzerland) and was of 0.981. TPA was uniformly distributed in 90-mm Petri plates that were placed in trays which were held in bags during the whole incubation period in order to

keep constant the relative humidity. A recipient with a glycerol solution adjusted at the corresponding a_w level was placed in the same tray to maintain this parameter constant throughout the experiment period. In the case of the plates used for RNA extraction, a porous cellophane disc (Packaging Limited, UK) was laid out over each of them.

2.3. Inoculation and incubation

Spore suspensions were prepared from 7-day-old PCA plates in 1 % peptone water for each strain and counted using a Thoma chamber. Two µL of a 10^6 *Alternaria* spp. spores/mL suspension were transferred to the centre of TPA plates. Plates were incubated at 4 different temperatures, 5, 12, 25 and 35 °C, for a maximum period of 35 days. These conditions were selected due to their relevance during postharvest management of tomato fruits as mentioned above. All combinations strain × temperature were assayed in triplicate.

2.4. Growth assessment

Radial growth was recorded daily by measuring two right-angled diameters during the incubation period or until the colony reached the edge of the plate. Colony diameter (mm) was plotted against the incubation time (days). Data plots showed, after a lag phase, a linear trend with time, thus linear model was applied. Lag phase prior to linear growth (λ, days) was determined as the interception between the regression line and the abscissa axis and maximum growth rate (μ_{max}, mm/day) as the slope from the linear growth phase.[26] A stationary phase followed the linear growth; in this phase no growth was observed while the fungus was still viable.

2.5. CWI-related gene expression

To study the effects of temperature on the response of the CWI pathway, the *RHO1* gene expression levels were evaluated at the linear and the stationary growth phases for each strain × temperature combination, in order to search for a relationship between this expression and fungal growth or mycotoxin production.[27,28] Samples were taken at different incubation times for each condition, based on growth data (Table 1). Mycelia were frozen in liquid nitrogen and ground in a frozen mortar to a fine powder. The extraction was carried out by Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's instructions (Protocol A). The RNA concentration and purity (A_{260}/A_{280} ratio) were spectrophotometrically determined using a 1.5 µL aliquot in a NanoDrop™ (Thermo Fisher Scientific, Wilmington, DE, USA). Samples were diluted to 0.1 µg/µL and treated with DNAse I (Thermo Fisher Scientific, Wilmington, DE, USA) to remove genomic DNA traces that could be co-extracted. After that, retrotranscription was carried out using PrimeScript™ RT reagent Kit (Takara Bio Inc., Kusatsu, Shiga, Japan) as described by manufacturer (incubation at 37 °C for 15 min and reverse transcriptase inactivation at 85 °C for 5 s). cDNA samples were stored at -20 °C until analysis. The real-time PCR reactions were performed in a ViiA™ 7 equipment (Applied Biosystems, Life Technologies, USA) using the SYBR™ Green technology, following the method described by da Cruz Cabral *et al*.[29] Briefly, the reaction mixture consisted of 6.25 µL of SYBR® Premix Ex Taq™ (Tli RNAseH Plus; Takara Bio Inc., Japan), 0.125 µL of ROX plus (Takara Bio Inc.), 200 nM of each primer (*RHO1*-F1/ *RHO1*-R2), 2.5 µL of DNA template and 3.125 µL of Milli-Q water. The thermal cycling conditions consisted of one holding period at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 55 °C for 1 min. After the final PCR cycle, the values of melting curves of the PCR products were checked to ensure the truthfulness of the results and specificity of the primers. Quantification cycle

(Cq), the cycle in which fluorescence reaches a defined threshold, was automatically obtained by the instrument using default parameters of the software ViiA™ 7 RUO v.1.2.4 (Applied Biosystems, Life Technologies, USA). Absolute expression levels of the *RHO1* gene of each sample were extrapolated from the standard curves by using Cq values obtained for each sample, as described by Rodríguez *et al.*[30]

2.6. Mycotoxin extraction and quantification

2.6.1. Extraction

Mycotoxins were analysed from TPA plates at the stationary growth phase for each condition, specified in Table 1. Extraction was performed following a QuEChERS methodology based on that proposed by da Cruz Cabral *et al.*[27] Nine agar plugs (7 mm-diameter each) were cut from the edge of the colony and dissolved in acetonitrile MS-grade acidified with 1% (v/v) formic acid after phase partitioning with aqueous salted solution. A 1 mL aliquot of the organic layer was taken and filtered through a 0.2 mm nylon filter (Jet Bio-Filtration Co., China) for subsequent analyses.

2.6.2. Detection and quantification

Extracts were analysed by an ultra-high-performance liquid chromatography–mass spectrometry (UHPLC–MS/MS) in a Dionex UltiMate 3000 HPLC system (Thermo Fisher Scientific, USA) coupled to an ion trap mass spectrometer model Amazon SL (Bruker Daltonics, Germany). UHPLC conditions used were those described in da Cruz Cabral *et al.*[27] MS analysis was conducted in ESI^+ by setting two time stages for scan, depending on the m/z ratio and retention time of the toxin: 0-7.50 min, scan range m/z 190-200 (TeA); 7.51-15.0 min scan range 268-278 (AME). Retention time for TeA was 7.3±0.2 min and for

AME was 10.1±0.2 min. Data processing was performed using the software DataAnalysis v4.1 (Bruker Daltonics) and peak areas were delimited manually.

2.6.3. Methodology performance

Standards of the two toxins were purchased from Sigma-Aldrich (USA). The calibration curves were constructed for quantification purposes by plotting peak areas versus toxin standard concentrations. Linear response range was established from this plot for each toxin (R^2 >0.99): 0.4-75.1 ng for TeA and 0.2-20.0 ng for AME. The limits of detection (LODs) were calculated from the calibration curves[31], being 72.5 ppb and 26.7 ppb for TeA and AME, respectively; while limits of quantification (LOQs) were 220.5 ppb for TeA and 81.1 ppb for AME. Recovery experiments were performed by spiking nine agar plugs previously weighed from TPA plates at three levels close to the minimum, medium and maximum points of the calibration curve for each toxin. Each level was evaluated in triplicate. The spiked plugs were incubated overnight at room temperature (20-25 °C) in order to allow toxins to absorb into the medium. Then, extraction and HPLC analysis were performed as described for the samples (sections 2.6.1 and 2.6.2). Mean recoveries of TeA and AME from triplicate samples were 77.6 and 93.8 % at the lowest level with coefficients of variation (RSDs) of 11.2 and 2.8 %, respectively; 70.0 and 105.6 % at the middle level with RSDs of 9.9 and 4.1 %, respectively; and 60.8 and 89.3% at the highest level with RSDs of 7.1 and 2.9 %, respectively. Average recoveries at the three levels of addition were 69.5 % for TeA and 96.3 % for AME. Quantities reported were corrected by recovery.

2.7. Statistical analysis

Statistical analysis was performed using the software IBM SPSS v.22.0 (IBM Corporation, USA). Data sets were tested for normality using the Shapiro-Wilk test. For normal and homoscedastic data according to the Levene test, an ANOVA was performed. Post-hoc comparison of means was made using the Bonferroni test. When data sets failed the normality test, the non-parametric Kruskal-Wallis test was applied. The Mann-Whitney U test was then selected for means comparison. The statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Effect of temperature on Alternaria *spp. growth*

Figure 1 shows an example of a growth curve obtained for *Alternaria* spp. on TPA plates at 12 ºC, indicating the selected times for RNA extraction and mycotoxin analyses. The mean lag phases (λ) and maximum growth rates (μ_{max}) of *Alternaria* spp. at each temperature are shown in Figure 2. Both strains presented similar behaviour regarding the relation of the growth parameters (λ and μ_{max}) with temperature, with minor interspecific differences.

With respect to λ, the shortest values for *A. arborescens* sp.-grp. were obtained at 25 and 35 ºC (< 1 day), while the largest was at 5 ºC (6 days). The longest λ for *A. tenuissima* sp.-grp. was also detected at 5 ºC (13.5 days), although for this strain, no significant differences were observed between the λ values at 35 ºC and 12 ºC and the minimum was obtained at 25 ºC (1 day).

For both strains, μ_{max} was detected at 25 ºC (7 and 10 mm/day for *A. arborescens* sp.-grp. and *A. tenuissima* sp.-grp., respectively). This parameter decreased rapidly when temperature was higher than the optimal (35 ºC), while a gradual decrease was detected at

lower temperatures. For *A. arborescens* sp.-grp., the minimum and maximum temperatures evaluated (5 and 35 °C) were equally unfavourable for this parameter, with μ_{max} = 2 mm/day. For *A. tenuissima* sp.-grp., the lowest μ_{max} was obtained at 35 °C.

3.2. Effect of temperature on Alternaria *spp.* RHO1 *gene expression*

The *RHO1* gene absolute expression for each condition assayed is shown in Figure 3. In the case of *A. arborescens* sp.-grp., at its linear growth phase, a maximum expression level of the *RHO1* gene was detected at 35 °C. The expression decreased with temperature, with the minimum level at the lowest temperature evaluated (5 °C). On the other hand, at the stationary phase, an opposite trend was noticed. The highest levels of the stress-related gene expression occurred at the lowest temperatures tested (5 and 12 °C), while the minimum number of copies was observed at 25 and 35 °C.

With respect to the *RHO1* gene expression by *A. tenuissima* sp.-grp. at the linear growth phase, no significant differences were detected among the 4 temperatures evaluated. However, at the stationary growth phase, the highest level of the *RHO1* expression was detected at 12 °C, while the lowest was observed at 35 °C.

3.3. Effect of temperature on Alternaria *spp. mycotoxin production*

The results of TeA production by *A. arborescens* sp.-grp. and AME by *A. tenuissima* sp.-grp. in TPA at different temperatures are shown in Figure 4. *A. arborescens* sp.-grp. synthesised large quantities of TeA at all temperatures tested, with an optimum production between 12 and 25 °C. The highest temperature studied (35 °C) was the least favourable

condition for this toxin biosynthesis. On the other hand, *A. tenuissima* sp.-grp. was able to produce AME only at the two lowest temperatures assayed; this toxin was not detected either at 25 or at 35 °C. The maximum production was obtained at 12 °C.

4. Discussion

A deep comprehension of the ecophysiological behaviour of mycotoxigenic fungi is a necessary first step in developing strategies to control food contamination. Additionally, it has been reported that some fungal proteins react upon changes in external conditions, leading to activation or inhibition of mycotoxin biosynthetic genes.[32] Given that little is known about the mechanisms by which external conditions may trigger mycotoxin biosynthesis in *Alternaria* spp., it is important to evaluate intracellular stress-related pathways in combination with the phenotypic metabolite production to understand the factors that lead to an increment of mycotoxin accumulation in food. Two *Alternaria* strains able to produce mycotoxins of different chemical structure each one were selected for this study. This allowed us to evaluate the effect of external conditions individually on each of them, considering that both mycotoxin biosynthetic routes are not completely elucidated yet and it is not known if both pathways are co-regulated at some level. In addition, these strains were also chosen because both were tomato pathogens, isolated from blackmould lesions. It is important to evaluate responses against stress in fungi from the food matrix under study, since the organisms may have acquired pre-adaptation mechanisms to the environments they are able to colonise.[18]

The present study was performed at 4 different temperatures that were selected due to their importance during postharvest management of tomato fruits: 5 °C represents a typical household storage temperature in refrigerator; 12 °C is a common temperature in storage chambers for industrialization; while 35 °C represents extreme temperatures that can occur in the field during the harvest of the fruits (Argentinean central region in high summer season). In addition, 25 °C was evaluated since it has been described as the optimum condition for *Alternaria* spp. growth[23] and it is an intermediate ambient temperature that can occur at postharvest stage and during fruit retailing.

There are few studies about small-spored *Alternaria* spp. growth and mycotoxin production under different environmental conditions, and some discrepancies are observed in the literature, probably associated with inter- and intra- specific differences. Regarding fungal growth rate, optimal temperatures were reported between 21 and 30 °C, in accordance with the results obtained in the present work.[3, 33, 34] Patriarca *et al*.[33] and Pose *et al*.[3] also reported a significant reduction in growth rate when temperature increased from the optimum, as it was observed in this work. The lag phase at 12°C, an average temperature in storing chambers, was much shorter than the normal time of storage of the fruits, which can be up to 14 days when are destined for fresh consumption, and several weeks for industrialisation. Consequently, the fungus will start active growth during storage under this condition, causing fruit decay and economic losses. Additionally, tomato fruits are sensitive to chilling damage, then it is not possible to use lower storage temperatures to delay fungal development. As spoiled fruits cannot be destined for fresh consumption, they are commonly used for industrialisation. Thus, tomato by-products may pose a higher

health risk since toxic fungal metabolites accumulated in the fruit are not destroyed by conventional heat treatments.

With respect to mycotoxin production, several studies agree that the range from 12 to 25 °C encompasses the optimal conditions for TeA biosynthesis, as observed in this work. Young *et al.* reported the maximum production at 20 °C by *A. tenuissima* in YES medium[35]; while Magan and Baxter observed the highest production levels at temperatures between 15 and 25 °C in modified Fries medium by *A. alternata* strains.[36] In addition, Pose *et al.* and Vaquera *et al.*, both works carried out in a tomato-based medium, detected the highest levels of TeA at 21 °C for *A. alternata* and 25 °C for *A. arborescens*.[25,37] It is worth highlighting that this mycotoxin was synthesised at all temperatures evaluated, indicating that in case of fruits contaminated with TeA producers, its accumulation could occur at any postharvest stage. On the other hand, AME was neither detected at 25 nor at 35 °C in the present work, whilst maximum production occurred at 12 °C. This result disagree with the scarce data available on small-spored *Alternaria* spp.; however the conditions evaluated in other works differ from those used in the present work, either in temperature, a_w or incubation period. The highest levels of such mycotoxin produced by *A. arborescens* was previously reported at 30 °C and 0.975 a_w.[25] Pose *et al*. detected the maximum accumulation of this mycotoxin at 0.954 a_w at 35 °C, while at 0.982 a_w, the highest level occurred at 21 °C after 7 days of incubation.[37]

External stimuli modulate metabolic and signalling pathways that affect the composition of the cell wall, which influences different processes depending on the development stage of the fungus, including growth, conidiation, mating, virulence and secondary metabolites production.[20] In the present study, the *RHO1* gene expression was evaluated at two

different growth phases for each strain, corresponding to the middle of the linear growth phase and at the beginning of the stationary phase. The *RHO1* gene expression at the linear growth phase allows evaluating the influence of CWI pathway on active fungal growth. Even though no clear relationship was observed among gene expression and growth parameters (λ and μ_{max}) as a function of temperature, both *Alternaria* strains showed higher levels of the expression of the *RHO1* gene as the temperature increased. This effect was more noticeable for the *A. arborescens* sp.-grp. strain. It has been reported for other fungal species, such as *Saccharomyces cerevisiae* and *Cryptococcus neoformans,* that an increase in temperature from 15 °C to 37 °C causes an activation of the CWI pathway due to an interrelation with the heat-stress response pathway through one of the membrane sensors capable of detecting temperature changes.[15,38,39]

At the stationary growth phase, the *RHO1* gene expression levels at the different temperatures showed no correlation with the production of TeA by *A. arborescens* sp.-grp., indicating that temperature would not be a link factor between these two intracellular pathways. Although little is known about TeA biosynthetic pathway and its regulation mechanisms, it has been previously shown that environmental parameters, such as a_w and temperature, influence on its accumulation.[25,37,40] This suggests that other stress-related pathways might be connected with its production.

On the other hand, for *A. tenuissima* sp.-grp., the *RHO1* gene expression at the stationary growth phase was significantly higher at 12 °C than at the remaining temperatures evaluated. This temperature was also at which the highest level of AME was produced, suggesting that both pathways could be correlated. The association between AME accumulation and *RHO1* gene expression has been recently reported in another work from

this research group in a different food-based matrix.[27] Moreover, this toxin biosynthetic route has been previously linked to other stress-related pathways in *Alternaria*, such as HOG (related to osmotic stress) and PacC (responses to pH changes).[18,41] Under specific environmental conditions, an increase in the transcription levels of these stress-pathways corresponded to a higher accumulation of alternariols. This interaction could be explained by the role of alternariols in nature as colonisation factors, for which a finely regulated biosynthesis is required. Additionally, the fact that the correlation with the CWI pathway was only detected for one of the mycotoxins studied is not striking, since it is well known that response mechanisms are not universal; the same stress-related route can act positively for a certain species and mycotoxin, and negatively, either for another species and the same toxin or another mycotoxin produced by the same species through a different biosynthetic pathway.[17]

5. Conclusions

Changes on temperatures related to different postharvest stages of tomato fruits have a marked effect on toxigenic *Alternaria* spp. TeA was strongly produced at all temperatures studied (5-35 ºC), whereas AME accumulation seems to be more important at lower temperatures of the range. Moreover, maximum levels of both toxins were detected at 12 °C; this is highly relevant, since it is a common storage temperature in chambers for industrialised tomato fruits. An association between the alternariols biosynthetic and CWI pathways was noticed in relation to temperature. Therefore, it seems that temperature may act as stressor stimulating the expression of a key gene of an intracellular stress-related pathway which in turn triggers this mycotoxin synthesis in *Alternaria* spp. These results are

important to understand the behaviour of this fungal contaminant in a susceptible matrix, tomato, which will be useful in developing new efficient strategies to control *Alternaria* mycotoxins in tomato fruit and tomato-based products (purées, sauces, concentrates), since temperature plays an important role at all postharvest stages from field to table. Of particular concern are processed by-products, for which storage of raw materials extends at temperature favourable for fungal growth and optimum for mycotoxin accumulation.

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Figure legends

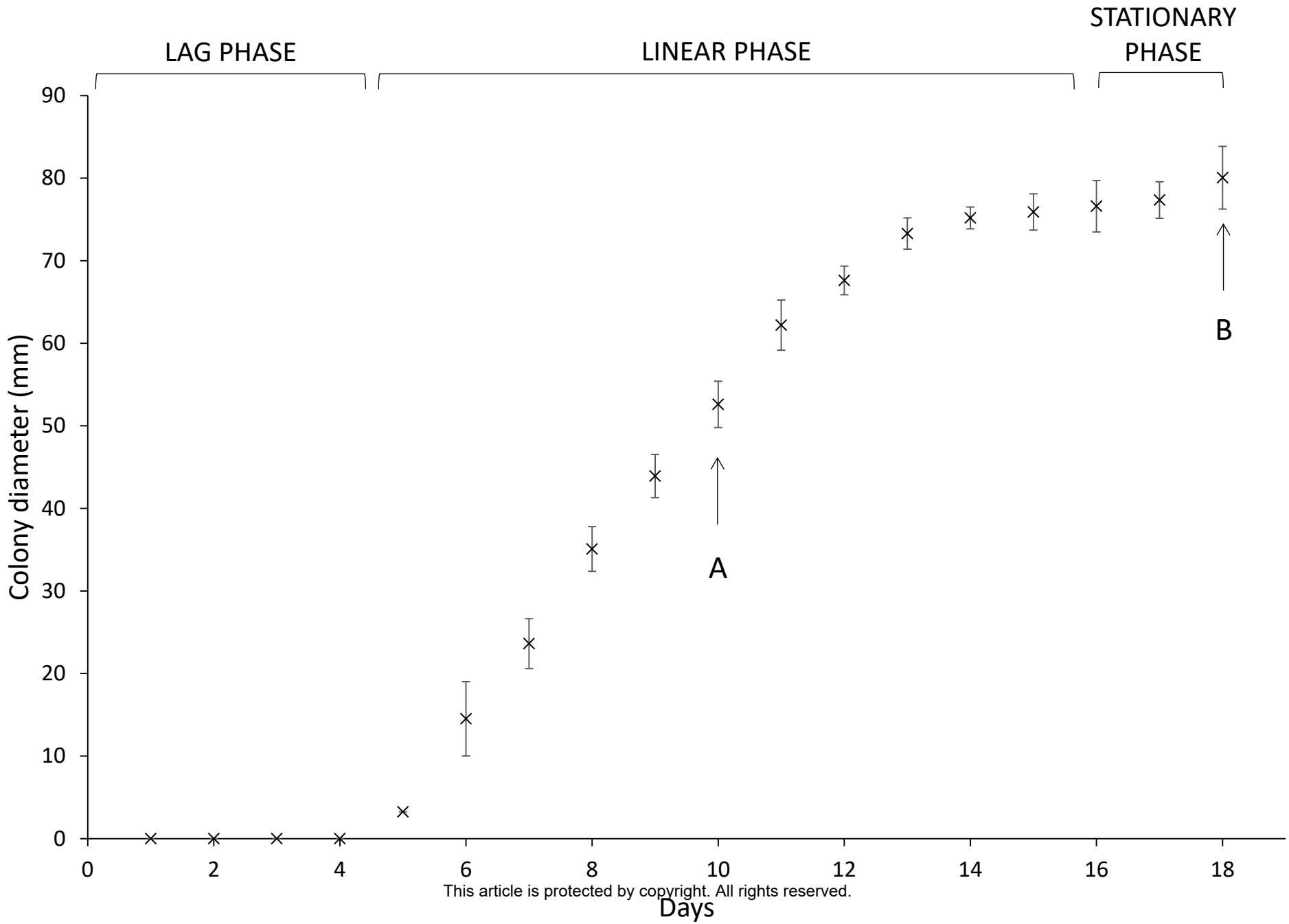
Figure 1. Growth curve of *Alternaria tenuissima* sp.-grp. at 12 °C on tomato pulp agar. Error bars indicate the standard deviation of measurements and arrows the days for RNA extraction (A and B) and mycotoxin extraction (B).

Figure 2. A) Lag phase (λ) and B) maximum growth rate (μ_{max}) of *Alternaria arborescens* sp.-grp.; C) λ and D) μ_{max} of *Alternaria tenuissima* sp.-grp.; at different temperatures (5, 12, 25 and 35 °C) on tomato pulp agar (TPA). Lines indicate the standard deviation and letters the groups with significant differences for each parameter of each strain ($p \leq 0.05$).

Figure 3. Absolute expression levels of the *RHO1* gene by *Alternaria arborescens* sp.-grp. (A) and *Alternaria tenuissima* sp.-grp. (B) in tomato pulp agar (TPA) at different temperatures (5, 12, 25 and 35 °C) and growth phases (linear and stationary). Lines indicate the standard deviation and letters the groups with significant differences for each strain ($p \leq 0.05$).

Figure 4. Production of tenuazonic acid (TeA) by *Alternaria arborescens* sp.-grp. (A), and of alternariol monomethyl ether (AME) by *Alternaria tenuissima* sp.-grp. (B) in tomato pulp agar (TPA) at different temperatures (5, 12, 25 and 35 °C) at the stationary growth phase. LOD: Limit of detection. Lines indicate the standard deviation and letters the groups

with significant differences for each strain ($p \leq 0.05$). Note different Y-axis range for both toxins.



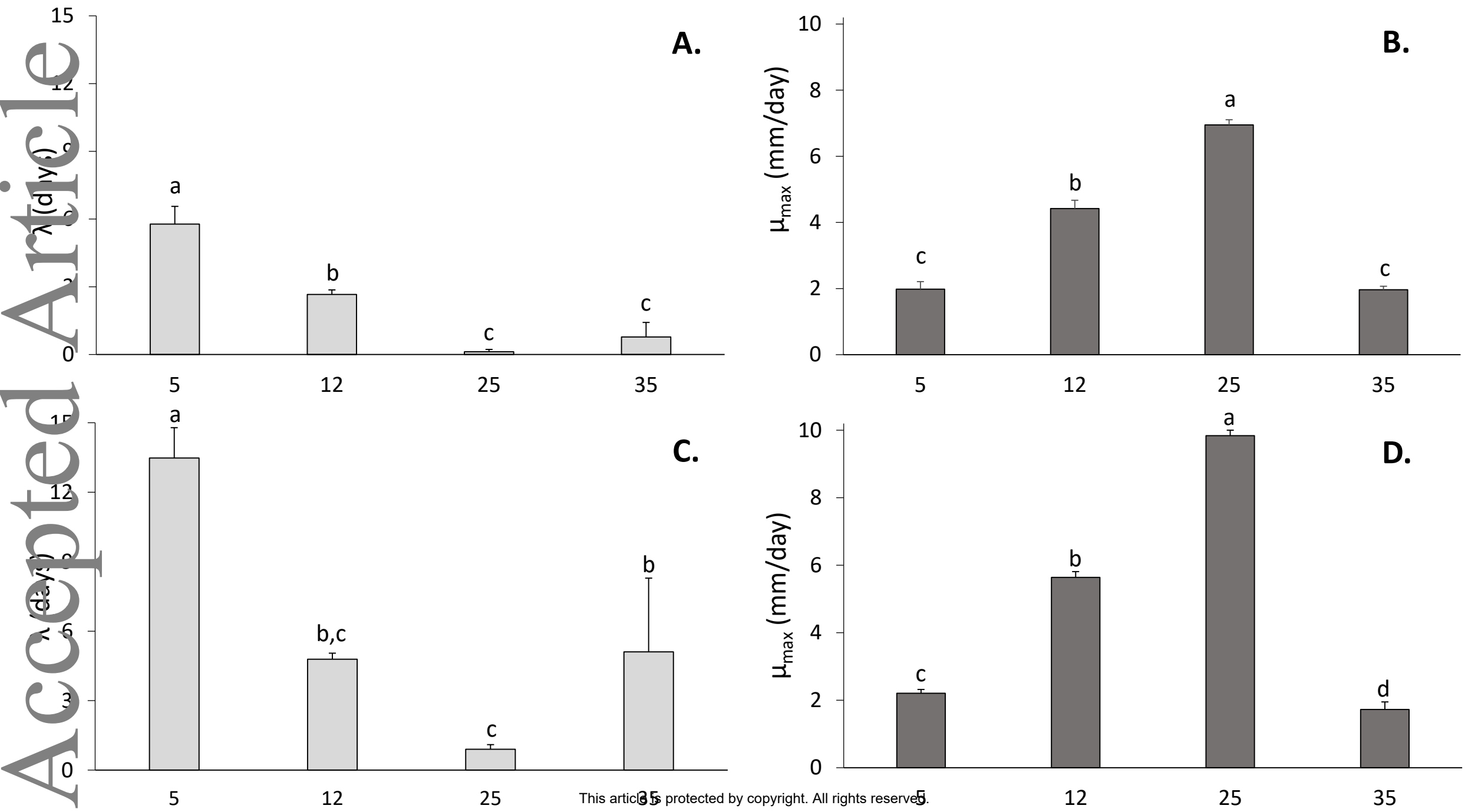


Figure 2

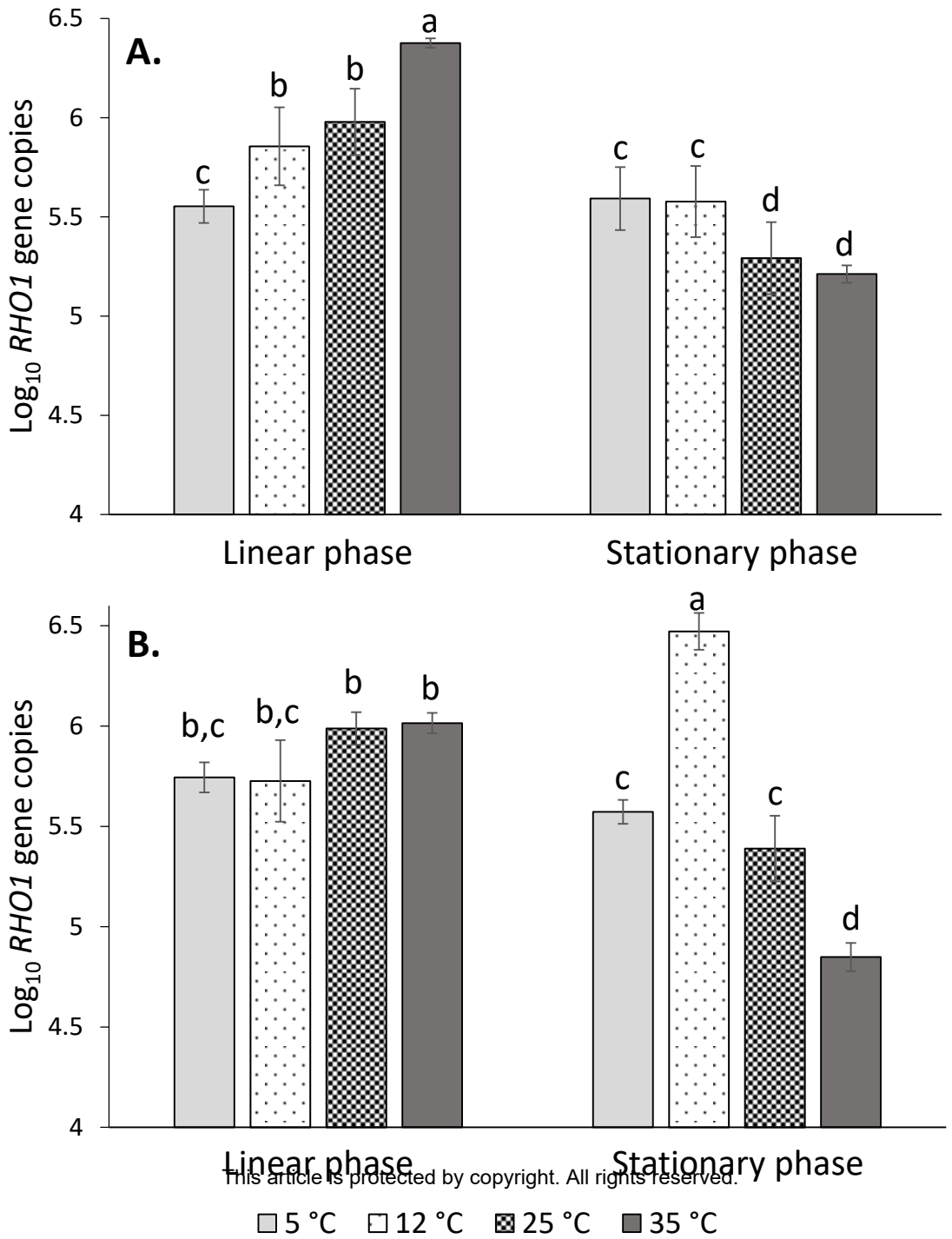


Figure 3

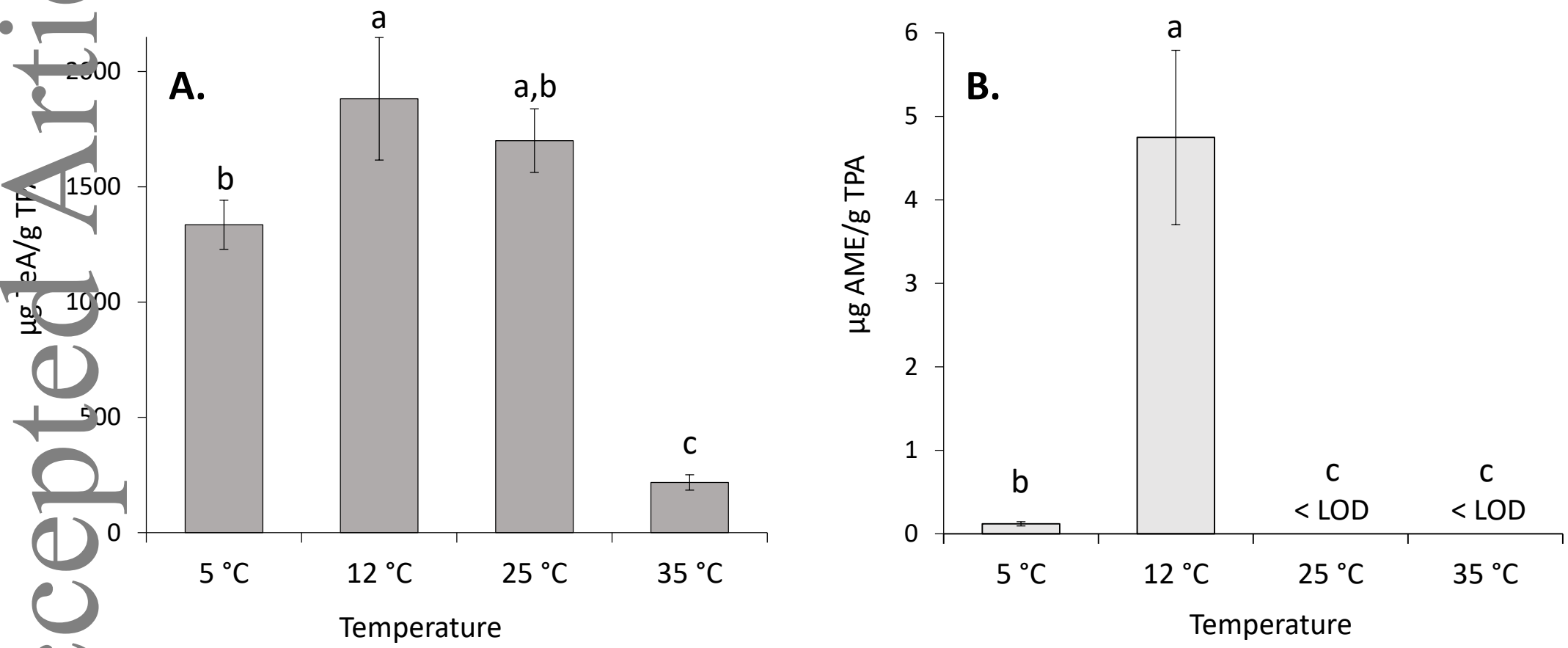


Figure 4

Table 1. Sampling days selected for the *RHO1* gene expression and mycotoxin extraction for *Alternaria* spp. at different temperatures (5, 12, 25 and 35 °C) on tomato pulp agar.

Alternaria spp.	Growth phase	Incubation time (days)			
		5 °C	12 °C	25 °C	35 °C
A. arborescens sp.-grp.	Linear	18	10	9	12
	Stationary	28	18	14	25
A. tenuissima sp.-grp.	Linear	25	10	9	18
	Stationary	35	18	12	28