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Utilizing low oxygen to mitigate resistance of stored product insects to phosphine

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Abstract

BACKGROUND: Data are provided on the utilization of modified atmospheres, at a commercial scale, against stored product insect populations that are resistant to phosphine. The method is evaluated on different populations of two major stored-product beetle species, *Rhyzopertha dominica* and *Oryzaephilus surinamensis*. The trials were carried out in commercial facilities, in which nitrogen was introduced through an embedded nitrogen generator. Each chamber contained three or four pallets of either currants or herbs. A computational model was developed to evaluate the nitrogen concentration.

RESULTS: In most trials, 100% mortality was recorded for both beetle species and all populations, regardless of the temperature and exposure intervals tested. Control progeny production ranged between 20 and 45 adults per vial for *R. dominica*, and 29 and 27 adults per vial for *O. surinamensis*. Simulation results reveal that nitrogen can easily penetrate the currants, and its concentration is uniform (differences are below 1.5%) across the pallet. Additionally, the simulation model revealed that lower temperatures do not have an impact on the nitrogen concentration profiles.

CONCLUSIONS: The modified atmosphere applications evaluated here were proved to be effective for all populations, regardless of the level of resistance to phosphine, and any survival could be attributed to the short exposure intervals. Modified atmosphere applications can be effective at a considerably short exposure interval, even at 2.5 days, which is an incontestable advantage for the use of this method against insects, at exposures comparable with those of commercial fumigations.
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Keywords: nitrogen; modified atmospheres; *Rhyzopertha dominica*; *Oryzaephilus surinamensis*; low oxygen; numerical modeling

INTRODUCTION

Modified atmospheres are considered as a highly promising method, which can be used with success for insect control at the post-harvest stages of durable agricultural commodities, as an alternative to traditional pesticides.[1-4] Considering the consumers' concerns for the risks of the use of residual insecticides to food, as well as the adverse effects of these insecticides on the environment, the use of modified atmospheres at the industrial level could offer a viable eco-friendly disinfestation strategy that can be applied in different commodities. The term 'modified atmospheres' does not correspond to one single application technique, as it refers to the change of the proportion of the gases in a given area (e.g. a chamber), and can be achieved through the artificial introduction of certain gases, such as nitrogen or carbon dioxide, to drastically reduce the concentration of oxygen or to increase the percentage of carbon dioxide.[4] The idea of the use of modified atmospheres is based on the fact that insects and most microbes are aerobic organisms, that require oxygen, and hence, the alternation of the atmosphere may have a catastrophic effect on their development and eventually their survival.[5-7] The application of modified atmospheres meets with many advantages, as it is a non-chemical, environmentally friendly method that leaves no residues on the treated commodity. Moreover, the mode of action of this method, through a physiological disorder of respiration, constitutes the development of resistance by the target organisms highly unlikely.[8]

Currently, the saturation of the atmosphere with nitrogen is probably more promising as compared with the addition of carbon dioxide, and nitrogen can be introduced in the target area using atmospheric air through generators.[9,10] Earlier studies, on the use of nitrogen, have shown promising results against many stored-product insect species.[11-17] For instance, Ofuya and Reichmuth[14] evaluated the effect of a 100% nitrogen atmosphere in all life stages of the cowpea weevil, *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) and the bean weevil, *Acanthoscelides obtectus* (Say) (Coleoptera: Bruchidae), and reported 100% mortality within exposure intervals that range between 1 and 9 days.

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Previous works have shown that stored-product insects can survive extremely low oxygen levels for long intervals, that exceed days or weeks,[18-20] and for this reason it has been suggested that the required oxygen levels for a satisfactory level of insect control should be lower than 2%.[1-3,21]

Even though the use of modified atmospheres is established at a commercial level and its efficacy is well-proven, its utilization for durable commodities by the industry is still limited. There are several studies on the efficacy of modified atmospheres in laboratory bioassays, but there are disproportionally fewer data regarding the evaluation of this method in large-scale commercial tests. The most used practice in applying modified atmospheres is the addition of nitrogen to reduce the oxygen content down to 1%, or even lower.[4,9,22] More recent data have shown that low oxygen, through the increase of the percentage of nitrogen, could be used with success for the control of insect populations that are resistant to the fumigant phosphine, and thus, this technique can be incorporated in phosphine resistance mitigation strategies.[22] Modified atmospheres can be applied directly to the commodity, which is not the common method for application of other methods that are mostly applied to the facilities, such as heat.[23] The first study that has evaluated nitrogen as a 'resistance breaker' for populations of the saw-toothed grain beetle, *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae), the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) and the rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) is that of Sakka *et al.*[22] In that work, the authors reported that all populations were found to be susceptible to nitrogen, regardless of their resistance level to phosphine.[22] However, most of the data available are based on bioassays that are carried out in chambers, but there is still inadequate information on the penetration of nitrogen into packaged products, such as pallets, and the 'speed of kill' in durable commodities. The implementation of numerical modeling has been used to some extent in similar applications, but we were unable to find studies relevant to the parameters of the present work. In the study of Guo *et al.*[24] numerical simulation results are shown for a case of liquid nitrogen injection in a container, suggesting that controlled atmosphere treatments are suitable and applicable for maintaining the quality of mature pepino fruit. Carvalho *et al.*[25] also studied nitrogen gas concentration for refrigeration and atmosphere modification in corn storage. In a similar approach, Silva *et al.*[26] performed Computational Fluid Dynamics (CFD) simulations of ozone gas for the control of the maize weevil, *Sitophilus zeamais* (Motschulsky) (Coleoptera: Curculionidae) in rice grains. Furthermore, Pandiselvam *et al.*[27] used numerical simulations to predict ozone concentration and flow characteristics in bulked paddy rice. By using CFD, Agrafioti *et al.*[28,29] modeled the distribution of phosphine gas inside grain silos and metal containers and validated this model in realworld scenarios.[30]

Based on the earlier, modified atmospheres could be further evaluated towards this direction, as a method to control insect populations that are resistant to phosphine, a phenomenon that has already taken global dimensions.[31] Therefore, in order to provide data on the utilization of modified atmospheres at a commercial scale against stored product insect populations that are resistant to phosphine, we evaluated this method on different populations of two major stored-product beetle species, the lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae), for which there are no relative data available, and *O. surinamensis*.

Furthermore, we developed a computational model to evaluate the nitrogen concentration of the treated commodities inside the chamber. The implementation of numerical modeling has been used to some extent in similar applications, but we were unable to find studies relevant to the parameters of the present work. For example, Silva *et al.*[26] performed CFD simulations of ozone gas for controlling the maize weevil, *S. zeamais* (Motschulsky) (Coleoptera: Curculionidae) in rice grains. Pandiselvam *et al.*[27] used numerical simulations to predict ozone concentration and flow characteristics in paddy bulk. Another example is the numerical simulations of Agrafioti *et al.*[28,29] on the distribution of phosphine gas inside grain silos and metal containers. In the study of Guo *et al.*[24] numerical simulation results are shown for a case of liquid nitrogen injection in a container. They concluded that controlled atmosphere treatments are suitable and applicable for maintaining the quality of mature pepino fruit. Carvalho *et al.*[25] also studied numerically the nitrogen gas concentration for refrigeration and atmosphere modification in corn storage.

MATERIALS AND METHODS

Insects

Both species are reared at the Laboratory of Entomology and Agricultural Zoology (LEAZ), Department of Agriculture, Crop Production and Rural Environment, University of Thessaly, Greece, in controlled conditions at 25 °C, 65% relative humidity (r.h.) with darkness. The preferable rearing for each species was soft wheat and oat flakes, for *R. dominica* and *O. surinamensis*, respectively

There were two populations from each species, one susceptible and one resistant to phosphine. The susceptible ones were the standard laboratory populations, which have been kept in LEAZ for several decades, and the resistant populations labeled as *R. dominica* GA6 and *O. surinamensis* ASC11. The susceptibility and resistance of the earlier populations have been evaluated in a recent study by Agrafioti *et al.*[32]

Bioassay protocol

Plastic cylindrical vials (3 cm in diameter, 8 cm in height, Rotilabo sample tins snap on lid; Carl Roth, Karlsruhe, Germany) that contained ten adults of the earlier species each, were the experimental units of our tests. Each vial had 10 g of commodity, which was soft wheat for *R. dominica* and oat flakes for *O. surinamensis*, with different series of vials for each species. In each trial, the vials were placed at different locations within the chamber (see later). For each species and population, there were three vials for each location. Additional series of vials were placed outside of the treated area, in the open air, and served as controls. After the termination of each trial, the vials were transferred at the facilities of the LEAZ, where adult mortality was recorded. Then, all vials were placed in incubators set at 25 °C, 65% r.h. and continuous darkness, while progeny production was recorded 65 days later.

Commercial trials

The trials were carried out in commercial chambers (237.6 m^3; AgroSpeCom, Thessaloniki, Greece), between January and August 2020, on which nitrogen was introduced through an embedded nitrogen generator, as described by Sakka *et al.*[22] In brief, nitrogen of high purity (99.1% nitrogen and 0.9% oxygen) was generated from the air through absorption-vacillation of pressure, and was introduced into the chambers at a maximum flow rate of 72 m^3 h^{-1}, while the temperature and the oxygen level were recorded continuously in all trials, with embedded sensors.[22] Each chamber contained three or four pallets of either currants or herbs, while all chambers had the capacity to maintain the

Table 1. Characteristics of the commercial trials

Trial number	Commodity	Month of the trial[a]	Temperature (°C)	Days of exposure[b]	Locations with vials
1	Currants	February	31	2	4
2	Currants	March	40	2.5	4
3	Herbs	March	28	6	4
4	Herbs	April	28	9	4
5	Herbs	April	28	9	4
6	Currants	May	28	3	4
7	Currants	July	28	9	4
8	Currants	June	28	9	4
9	Herbs	July	28	9	4

[a] Indicates the month on which the commercial trial took place.
[b] Calculated as number of days after the interval on which the desired temperature was achieved.

temperature at the desired level. The vials were placed in all pallets within the chamber, in different locations, i.e. under the pallet, at the core of the pallet, or at the upper part of the pallet, as described by Athanassiou *et al.*[9] In total, there were nine commercial trials, with different temperatures and exposure intervals, as illustrated in Table 1, while in all cases oxygen level was maintained at ≤ 1%.

Data analysis

All data, separately for each trial and insect species, were subjected to a *t*-test at 0.05, with insect mortality as the dependent variable and the insect population as the main effect. For the evaluation of the effect of location within each trial, separately for each species, the data were submitted to one-way analysis of variance (ANOVA), with insect mortality as the response variable and location as the main effect. Control mortality was not included in the analysis, as it was negligible. The same approach was used with progeny production counts. Means were separated by the *t*-test at 0.05. The SPSS version 25.0 software (SPSS Inc., Chicago, IL, USA) was used for all analyses.

Numerical simulations

The initiation of each protocol started when the oxygen concentration in the chamber (outside the pallets) reached values below 1% (or nitrogen to 99%). To further strengthen the analysis of the present study, a numerical model is developed to provide predictions of the nitrogen concentration of the treated commodity. Under the assumption that the nitrogen concentration surrounding the pallets is uniform and that the main mechanism of nitrogen gas distribution inside the pallet is gas diffusion, the equation that needs to be solved is:

$$\frac{\partial G}{\partial t} = D_{m,\mathrm{eff}} \nabla G^2$$

where G is the nitrogen gas concentration, t is the time (in seconds), and $D_{\mathrm{m,eff}}$ is the effective diffusion coefficient (in $\mathrm{m}^2\ \mathrm{s}^{-1}$) which considers the porous effects of the currants to the gas flow. According to Neale and Nader,[33] the effective diffusivity with respect to porosity is $D_{\mathrm{m,eff}} = 0.24\ D_{\mathrm{m}}$. The porosity value of the currants is 40%.[34] Concerning the binary (nitrogen/oxygen) diffusion coefficient (D_{m}), it was calculated based on the equation originating from the molecular theory of gases.[35] Thus, D_{m} is a function of temperature, and for the present calculations the value was 2.076E-05 and 2.186E-05 for temperatures of 28 and 40 °C, respectively.

The interaction of the computational domain (pallet) with its surroundings (chamber) is implemented in the simulation using the corresponding boundary conditions. For the present study, the gas concentration on the boundaries was spatially uniform and varied in time. The time variation corresponds to a typical case, retrieved from sensor data and is shown in Fig. 3 (see later).

Meshing is the discrete representation of the geometry that is involved in the problem. Essentially, it partitions space into cells over which the equation can be approximate. In the present study, the computational grid used was structured ($80 \times 107 \times 80 = 684\,800$ cells), thus ensuring greater accuracy. Furthermore, grid-clustering was employed near the side to properly capture large gradients. The simulation process, as described earlier, was implemented in OpenFOAM[36] and the visualizations in Paraview.[37]

Since the sensors were placed outside the pallets, the goal of the numerical procedure is to evaluate the concentration of nitrogen at every point inside the pallet, at every time step of the treatment. Thus, the concentration at the core of the pallet (it is expected to be the most difficult point) and the effect of temperature on the diffusion coefficient can be known.

RESULTS

Adult mortality

In most trials, we recorded 100% mortality for both tested beetle species, and all populations, regardless of the temperature and exposure intervals tested (Table 2). The only exception was Trial 1, where, although all *O. surinamensis* adults were dead, we recorded an extremely high adult survival of adults of both *R. dominica* populations. However, there were no significant differences in the mortality levels between *R. dominica* GA6 and *R. dominica* Lab. In the case of the control vials, adult mortality was negligible, and therefore control mortality data were not included in the analysis.

Progeny production

Control progeny production ranged between 20 and 45 adults per vial for *R. dominica* GA6 and *R. dominica* Lab, respectively, while the corresponding figures were 29 and 27 adults per vial for *O. surinamensis* ASC11 and *O. surinamensis* Lab, respectively. No progeny production was recorded in the treated substrate, except for

Table 2. Mean parental mortality (% ± standard error) of adult beetles for each species and populations in each trial, along with the progeny production counts (number of individuals ± standard error), and the range of values among locations for both mortality and progeny

Trial number	Species/population	Adult mortality	Range of adult mortality among locations	Progeny production	Range of progeny production among locations
1	*O. surinamensis* ASC11	100 ± 0.0	0.0–0.0	1.4 ± 0.7	0.0–5.6
	O. surinamensis Lab[a]	100 ± 0.0	0.0–0.0	1.1 ± 0.7	0.0–4.3
	R. dominica GA6	5.0 ± 2.3	0.0–6.6	16.5 ± 1.7	14.3–19.3
	R. dominica Lab	5.8 ± 1.9	3.3–6.6	16.6 ± 0.7	15.0–17.6
2	*O. surinamensis* ASC11	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
	O. surinamensis Lab	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
	R. dominica GA6	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
	R. dominica Lab	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
3	*O. surinamensis* ASC11	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
	O. surinamensis Lab	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
	R. dominica GA6	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
	R. dominica Lab	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
4	*O. surinamensis* ASC11	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
	O. surinamensis Lab	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
	R. dominica GA6	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
	R. dominica Lab	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
5	*O. surinamensis* ASC11	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
	O. surinamensis Lab	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
	R. dominica GA6	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
	R. dominica Lab	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
6	*O. surinamensis* ASC11	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
	O. surinamensis Lab	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
	R. dominica GA6	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
	R. dominica Lab	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
7	*O. surinamensis* ASC11	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
	O. surinamensis Lab	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
	R. dominica GA6	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
	R. dominica Lab	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
8	*O. surinamensis* ASC11	100 ± 0.0	0.0–0.0	2.0 ± 2.0	0.0–8.3
	O. surinamensis Lab	100 ± 0.0	0.0–0.0	0.2 ± 0.2	0.0–1.0
	R. dominica GA6	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
	R. dominica Lab	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
9	*O. surinamensis* ASC11	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
	O. surinamensis Lab	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
	R. dominica GA6	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0
	R. dominica Lab	100 ± 0.0	0.0–0.0	0.0 ± 0.0	0.0–0.0

Within each commercial trial and each species according to Student's *t*-test parameters for adult mortality were: in Trial 1 for *Rhyzopertha dominica* $t = -0.277$, $P = 0.784$.

According to *t*-test, the parameters for *progeny production* were: in Trial 1 for *Oryzaephilus surinamensis* $t = 0.236$, $P = 0.816$, for *R. dominica* $t = -0.087$, $P = 0.932$, in Trial 8 for *O. surinamensis* $t = 0.874$, $P = 0.400$, with df = 11.31. In all cases df = 22. No significant differences were noted, since no letters exist.

[a] 'Lab' indicates phosphine susceptible insect populations.

Trials 1 and 8 (Table 2). For Trial 1, progeny production was noted in the vials that contained *R. dominica*, where parental mortality was high, but also in the vials that contained *O. surinamensis*, where there was no parental survival. Moreover, in that trial, adult emergence was high in the case of *R. dominica* and in all vials that had been placed within the chamber, while progeny production for *O. surinamensis* was recorded only in some locations. In contrast, progeny production in Trial 8 was recorded only for *O. surinamensis*, and only in one of the locations tested, while there were no significant differences between *O. surinamensis* ASC11 and *O. surinamensis* Lab, or among locations (Table 2). An overview of the results of Table 2 is shown in Fig. 1, where a scatter plot of the 'Progeny Production' for each species is shown.

Computational results

The simulation model was used to predict the nitrogen concentration inside a pallet loaded with currants, at 40 °C. Figure 2 shows the two-dimensional spatial profile under typical boundary conditions for three instances (1, 3, and 8 days). Additionally, the concentration at a horizontal line (at mid-height) is shown. As expected, higher concentrations are located near the outer regions of the palette, whereas the core of the palette has the minimum concentration during the treatment. Figure 3 shows the time evolution of the nitrogen concentration at the palette core and on the boundary and helps to evaluate the concentration difference between those two regions. The simulation results reveal that the concentration difference does not exceed 1.5%,

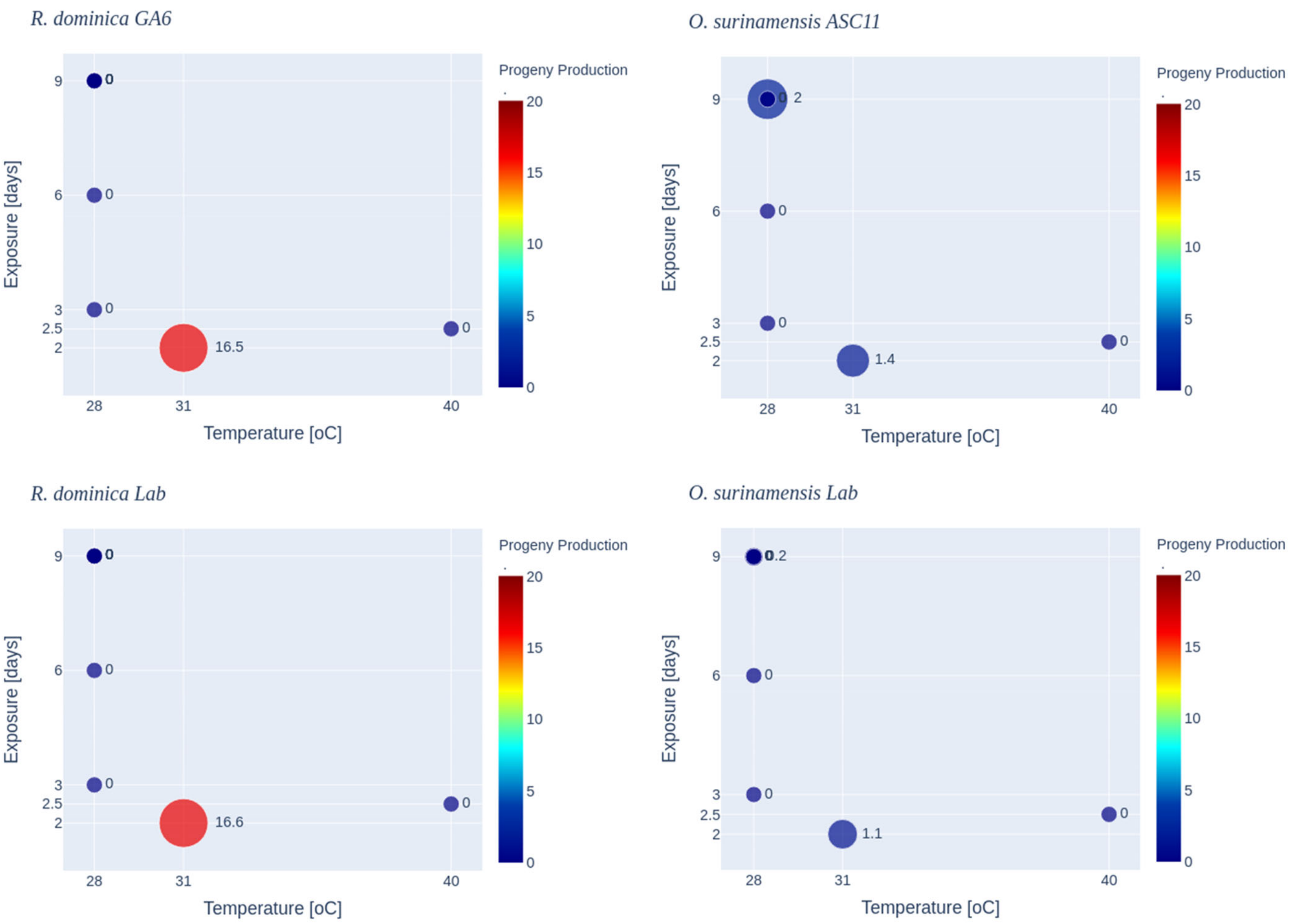

Figure 1. Scatter plot of the progeny production for each species. The coordinates of each point correspond to the conditions (temperature and exposure) of each trial, while the size and color of the points, correspond to the progeny production.

and the largest difference is found in the early stages of the treatment. The initiation of the treatment protocol takes place when the nitrogen concentration reaches 99% (or oxygen concentration 1%). According to the sensor data (which were placed on top of the pallet), for a typical case, this status occurs near the tenth day. Simulation results reveal that nitrogen can easily penetrate the currants, and its concentration is mostly uniform (differences are below 1.5%) across the pallet.

We performed a similar simulation for a case with a lower temperature (28 °C) to evaluate the effect of the lower diffusion coefficient. The results showed that a temperature difference of 12 °C does not have a significant effect on the nitrogen concentration and the results are similar to the 40 °C (thus no figures are included in this study).

DISCUSSION

As indicated earlier, the use of modified atmospheres for the control of stored-product insects has been tested in different application scenarios, since the data on the use of this method on a commercial scale is rather limited.[12-16,38] Moreover, the data available so far are also widely focused on the effects of this method in certain qualitative characteristics of specific low-moisture commodities, such as grains and legumes.[39] In our case, we have tested durable ‘high value’ commodities, such as currants and herbs, illustrating the wide applicability of the method and its effectiveness against insects to a wide range of products. For currants, Athanassiou *et al.*[9] noted that the application of nitrogen, apart from its high efficacy against stored-product insects, reduced the microbiological load, without affecting the basic product's qualitative characteristics.

We used *O. surinamensis* and *R. dominica*, as these are common species that have been often found in a wide range of stored product in Greece,[40,41] while a recent study underlined the occurrence of resistance in populations of these two species sampled from Greek storage and processing facilities.[32] Our results demonstrate that resistance to phosphine is not related with the insecticidal effects of nitrogen, as there were no differences in parental survival and progeny production between the two populations used for both species. Similar results have been also recorded by Sakka *et al.*[22] for different stored-product beetle species, but this is the first study that has examined different populations of *R. dominica*, under this experimental scenario. For this species, we saw similar susceptibility levels between the two populations, which stands in accordance with the results for other major stored-product beetle species reported by Sakka *et al.*[22] Hence, modified atmospheres can be used with success for the control of stored-product insect populations that are resistant to

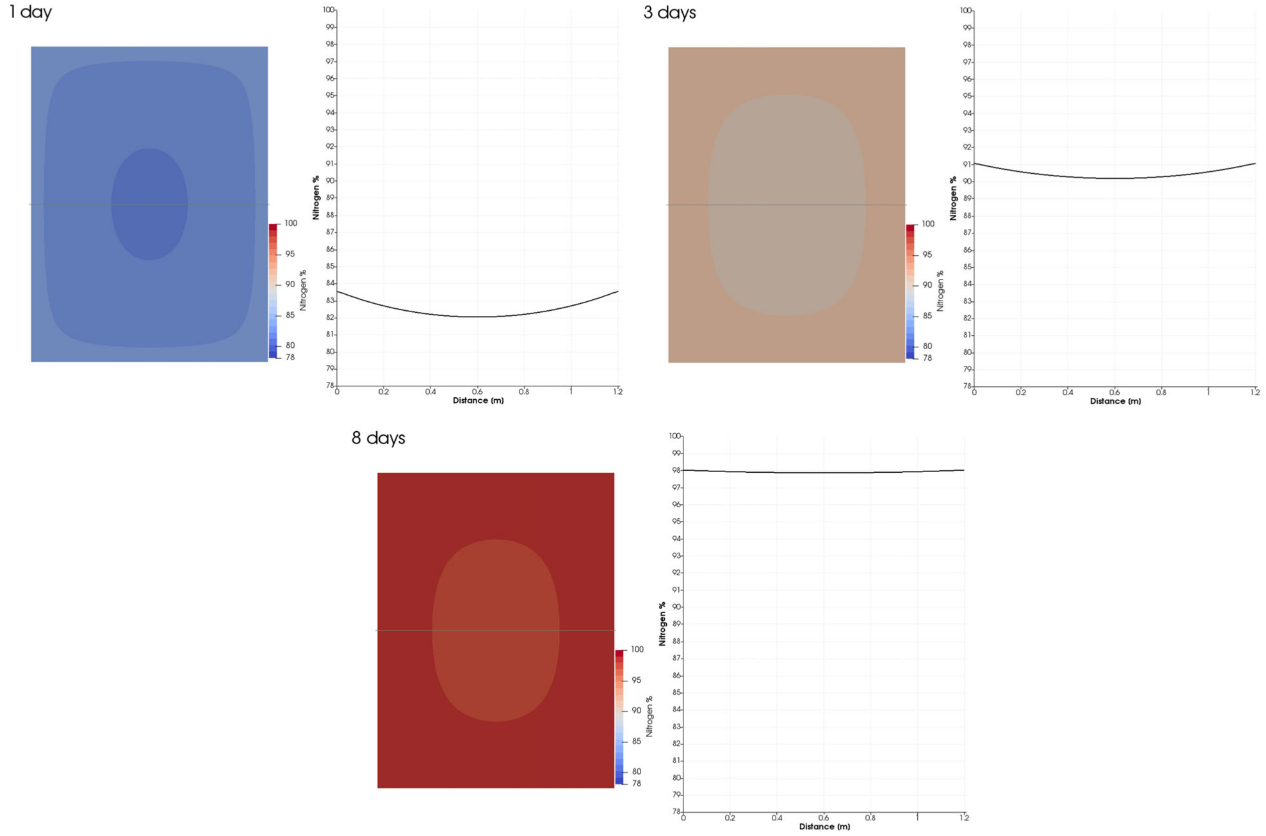

Figure 2. Nitrogen concentration profiles for a palette loaded with currants, under typical boundary conditions for three instances (1, 3, and 8 days). On the right a two-dimensional representation is shown, whereas the concentration at a horizontal line (at mid-height) is shown.

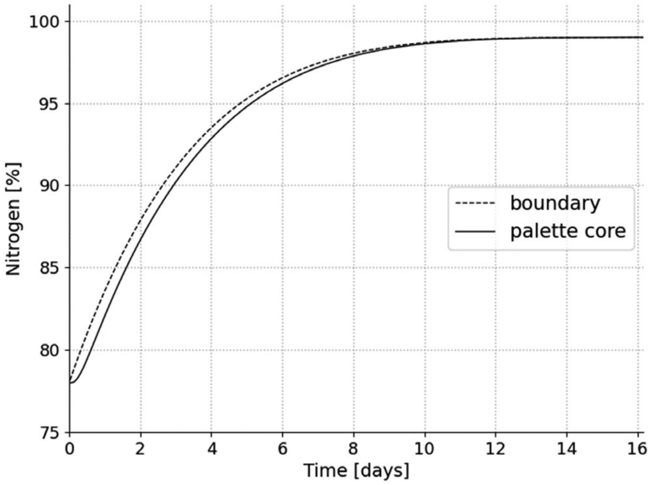

Figure 3. The time evolution of nitrogen concentration at the palette core (solid line) and the boundary conditions (dashed line).

phosphine as an alternative treatment method. Moreover, we demonstrate that this can be achieved at exposure intervals that are comparable with those that are used for phosphine.[28-30]

Earlier studies have partially quantified the effect of temperature on the insecticidal effect of modified atmospheres which are based on the use of nitrogen.[42-44] All of these works concur that stored-product insect mortality is higher at elevated temperature levels, which could be attributed to the acceleration of the metabolism, as well as increased stress.[23,45,46] For instance, Soderstrom *et al.*[42] reported that a temperature level as high as 38 °C in conjunction with the increased percentage of nitrogen in the atmosphere increased larval mortality of *T. castaneum*, and drastically shortened the overall exposure period. Moreover, Chiappini *et al.*[44] demonstrated that there is a negative correlation between temperature and the required insect exposure to nitrogen, i.e. the higher the temperature, the shorter the treatment. In that study, the authors examined the exposure of larvae of the confused flour beetle, *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae) at 38–43 °C and found very high mortality levels after 1–2 days of exposure.[44] At the same temperature range (38–43 °C), Athanassiou *et al.*[9] found that mortality of all life stages of different stored-product insects in commercial nitrogen chambers could be shortened to 3 days. Thus, although traditionally modified atmospheres were considered as a slow-acting control method,[4] that required several weeks to act, newer data indicate that this interval can be sufficiently shorter when nitrogen is combined with increased temperatures,[9,22] and as a result, any algorithms that deal with the application of nitrogen in chambers should be recalculated towards this direction. Apparently, this requires the capacity of a given chamber to be heated, but most modern commercial types of chambers that are currently in use are equipped with temperature control equipment.[22]

We have tested the application of nitrogen through atmospheric air in chambers, which is the best-case scenario, as nitrogen can easily replace oxygen in such a confined area, where apparently, the increase in temperature may also accelerate gas distribution, in terms of a more rapid replacement of oxygen by nitrogen. Recent studies have shown that fumigation with phosphine is more likely to be successful in small spaces, such as shipping containers, as compared to larger storage facilities, such as large warehouses and vertical silos.[29,30,46] For instance, in a wide screening of different commercial fumigations with phosphine, Agrafioti *et al*.[30] reported that the majority of the fumigations in containers were successful, while insect survival was high in many of the fumigations in larger facilities. In this context, chambers are not the only facility where nitrogen can be applied, and earlier studies have shown that similar generators can be used in larger spaces, such as concrete silos.[4,39] In a commercial application in silos in Cyprus, Navarro[4] reported that the treatment required more than one week or even two weeks, and even in this case, insect survival could not be avoided. In these facilities, additional sealing improvements should be performed, in contrast with the treatment in chambers, where it is usually taken for granted that serious leakages are less likely to occur. At the same time, even after long treatments, 'oxygen nests' can be present in large storage facilities, which necessitates the expansion of the treatment period, to minimize the spots that may allow insect survival. Moreover, in contrast with the application in chambers, although doable, it is not feasible to heat the treated substrate in large storage facilities, and thus, it is not possible to accelerate the treatment interval through heat. Thus, the temperature-exposure data that are reported here correspond to chambers and are not transferable in larger facilities.

The shortened treatment periods should be considered as an additional time to the initial time that is required to increase the temperature to the desired level. This initial time, however, is not stable and depends on the initial temperature that prevails during the initiation of the application. Consequently, if the application is carried out during the cold period of the year, it is expected that the increase of temperature to the desired level will take longer, as compared with a treatment that is carried out during the warm period of the year. This might be a concern in terms of the economic feasibility of heating in countries that have a climate that is colder than that in Greece, but the overall cost could be notably moderated by the shortened treatment duration. However, the short duration of Trial 1 was the reason for the increased survival and progeny production of *R. dominica*, but, in order to reach 31 °C which was the target temperature for this specific treatment, several days need to be added before the 2-day treatment. Similarly, the trials that were carried out during the summer months probably required a very short 'pre-conditioning' period, and they were effective at temperatures that were as low as 28 °C. Considering the overall data reported here, we have strong indications that exposure is more important than temperature and that additional exposure days may alleviate the negative effects of low temperatures. Conversely, if there are time limitations, i.e. a given application should be completed in a short period of time, the increase of temperature from 31 to 40 °C may increase insect morality, if it is combined with a small increase of the exposure time (from 2 to 2.5 days). Nevertheless, higher temperatures may not be desirable, due to possible detrimental effects on certain physicochemical characteristics that are to be treated.[9] The pre-conditioning interval may be a factor of either positive or negative effect on the exposed insects, prior to the application of nitrogen, through additional stress or additional hardening, but this remains to be further investigated. Athanassiou and Arthur[8] found that, in general, the 'cool down' of stored product beetles before exposure to low temperatures (0 °C) and the subsequent 'warm up' after the termination of the exposure, did not result in any difference as compared with insects that were not exposed to 'cool down–warm up' conditioning. To further strengthen our analysis, we performed numerical simulations to predict the nitrogen concentration on the entire pallet, which verified the spatial uniformity as mentioned earlier. Additionally, the simulation results showed that temperature differences (up to 12 °C) do not have an impact on the speed of nitrogen penetration. In other words, our model showed that the increase in temperature practically had no effect on the speed of nitrogen penetration within the pallet, and thus, any increase in mortality should be regarded as a direct consequence of insect stress.

Modified atmospheres have been proven to have a good penetration ability into the pallets, given that control levels were comparable to all locations tested. This is particularly important, as in the central part of the pallet (the 'heart') airflow is limited and oxygen nests may exist. Similar results have been also reported in previous studies, with different experimental protocols.[1,22] In most trials, the application resulted in complete mortality of the exposed individuals of all life stages, including the eggs. Other gases, such as ozone[47,48] and sulfuryl fluoride[49,50] are not considered very effective against eggs of stored-product insects.

In summary, the modified atmosphere applications evaluated here were proved to be very effective for all populations, regardless of the level of resistance to phosphine, and any survival could be attributed to the short exposure interval. The results of the present work show that modified atmospheres that are based on the use of nitrogen can be effective at a very short exposure interval, even at 2.5 days, which is an incontestable advantage for the use of this method against insects. We also demonstrated a clear positive effect of temperature to the overall duration of the application, which is not always possible with other gases that are used as insecticides. Finally, low oxygen treatment, through the increase of nitrogen percentage, has a good penetration ability in pallets, and a good distribution pattern within the chamber and the product mass, as it was confirmed by the simulations as well.

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