Effect of ozonation on table grapes preservation in cold storage

E. Vlassi¹ · P. Vlachos² · M. Kornaros²

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Abstract Table grapes artificially inoculated with *B. Cinerea* were tested under four different ozonation strategies in order to achieve prolongation of table grapes' shelf-life time. Decay incidence, external disease appearance, number of infected grapes, weight loss and a variety of quality parameters such as sugars and proteins content were checked after every 3 days. No significant alteration of table grapes quality characteristics was observed after their exposure to ozone atmosphere. Moreover, the low ozone dosage process (0.3 ppm) caused sufficient restriction of fruit decay due to fungal contamination and secured a 40-days-period of storage time. However, the observed weight loss was somewhat higher on the treated samples compared to the untreated ones. From technical and economical point of view the low dosage (0.3 ppm) ozonation process on a daily basis combined with the cold storage appears to a very promising method for table grapes preservation.

Keywords 'Sultanina' table grapes · Postharvest · *B. Cinerea* · Gray mould · Preservation · Ozone

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✉ E. Vlassi
vlassi.helena@gmail.com

¹ Theoretical and Physical Chemistry Institute, National Hellenic Research Foundation, 48 Vassileos Constantinou Avenue, 11635 Athens, Greece

² Department of Chemical Engineering, University of Patras, 1 Karatheodori St, 26500 Patras, Greece

Introduction

Ozone is triatomic oxygen, a naturally occurring form of oxygen that was first identified in 1840. Except from the second most powerful chemical oxidant readily available, it is an excellent sterilizing strong sanitation and fumigation agent. Because of these properties, ozone has been studied for preservation of foods in the storage room and during shipping to prevent bacteria, mold, and yeast on the food surface and to control insects. It can also eliminate undesirable flavor produced by bacteria and chemically remove ethylene gas to slow down the ripening process, thus allowing extended distribution (Rice et al. 1982).

Recently, the use of ozone in the production and preservation of foods has come in the forefront, due to the recent approval of the ozone usage as an antimicrobial agent for the treatment and storage of foods by the United States Food and Drug Administration (US-FDA) (Zhang et al. 2010; US-FDA 1997). The FDA approval constitutes a milestone for the food industry. In the mid-1990s, the use of ozone in food production had already been allowed in Japan, Australia, France and in other countries for a long time (Hampson and Fiori 1997; Xu 1999). Until today, various applications which have been related to the use of ozone in the production and preservation of foods, have been reported (Shankar et al. 2014), such as in the preservation of fish and shells (Gonçalves 2009), in the freezing of meat (Zhou et al. 2010) and in the disinfection and preservation of fruits and vegetables (Miller et al. 2013). After the abrogation of restrictive regulations about the use of ozone in the food industry, many studies have been conducted in order to determine the economical cost for the use of ozone.

It was not until 19th century when the production of table grapes became popular and economically important

playing an important role in the fresh market (Zsuzsanna and György 2012). Although they are non-climacteric fruits with a low rate of physiological activity, they are subject to serious water loss and decay during postharvest handling (Lichter et al. 2008). Since 1940 to 2011, Miller et al. (2013) recapitulated analytically the variation of results when berries of table grapes were treated with gaseous ozone exposure (e.g. continuous, intermittent). Different concentrations (450 ppb, 0.3, 2–3, 16, 200–350, 2500, 5000 and 10,000 ppm) of ozone have been reported in studies. Later on (2014), ozone at $C \geq 0.100 \mu\text{L/L}$ inhibited the spread of gray mold among stored grapes and the combination of ozone and (initial or in between these fumigations), controlled both gray mold and other pathogens. Since there were no detectable differences in flavor of grapes treated with ozone compared to those stored in air, grapes treated with ozone were preferred over those treated with sulphur dioxide (Klaasen et al. 2006). It is important to remember that considerable care needs to be taken to control the atmospheric concentration of the gas in stores/transit containers and the reaction to, and benefits of, ozone treatment vary between commodities of table grape.

Controlled atmosphere combining $\text{O}_2 + \text{CO}_2$ with continuous O_3 $0.1 \mu\text{L/L}$ (Artés-Hernández et al. 2004) and combining $\text{O}_2 + \text{CO}_2$ with continuous O_3 2 ppm (Artés-Hernández et al. 2007) (CA treatments) were used on white seedless table grapes for keeping their quality during cold storage. The first CA treatment (Artés-Hernández et al. 2004) mentioned not sufficient for commercial purposes and the second CA atmosphere treatment (Artés-Hernández et al. 2007) was found not to completely inhibit fungal development. In addition, a slight decrease of total phenol content was observed under CA atmosphere with continuous presence of 2 ppm ozone. In Palou et al. (2002) it is reported that, in similar ozonation conditions (continuous 0.3 ppm ozone treatment) for table grapes ('Thompson Seedless') the grey mould was limited completely, however this was not the case when the grapes had been previously contaminated via immersion. The aim of the current work was the table grapes preservation with the use of ozone. In details, the table grapes were subjected to artificial contamination with spores of *B. Cinerea* via two different ways and in the sequel an ozone treatment was followed. The study was focused on the extension of table grapes shelf-life using gaseous ozone as a disinfectant agent. The scope was to propose a replacement for fungicides in future applications as they have been proved extremely dangerous and pathogens for the environment and human health.

Materials and methods

Glassware

In order to prevent cross-microbial contamination of table grapes, all glassware used was sterilized in the autoclave (121°C , 30 min).

Chemicals and reagents

Analytical standards of glucose and albumin were of high purity ($> 98\%$) and were obtained from Sigma Chemical Corp. All chemicals were reagent grade or analytical grade, when available and were used without further purification. Water was prepared in a Milli-Q purification system (Millipore).

Tables grapes

Table grapes belong to Greek variety of Corinthian 'Sultantina' and were provided by a local producer (Kiato region, northern part of Corinthia in the Peloponnese, Greece).

B. Cinerea spores (solid cultures)

B. Cinerea is a classical "high-risk pathogen" and the exhaustive use of reduced-risk fungicides should be expected to lead to rapid selection of resistant isolates (Smilanick et al. 2010; Brent and Hollomon 2007). As a suitable medium for *B. Cinerea* cultivation the Potato Dextrose Agar (PDA) from Sigma Chemical Corp was selected. The ideal incubation temperature for *B. Cinerea* spores (Krause and Weidensaul 1977) on PDA medium is 20°C and the ideal incubation time varies between 7 and 14 days.

Reactor

An advanced ozone reactor system capable of operating at controlled temperature, humidity, pressure and ozone concentration conditions was constructed and is described in the sequel. Ozone is supplied to the reactor by a laboratory scale ozone generator (Prominent Ozon lab) provided by Prominent GmbH capable of producing $750 \text{ mgO}_3/\text{h}$ operating at 50 L/h and about $175 \text{ mgO}_3/\text{h}$ operating at 10 L/h using ambient pure oxygen. The ozone produced by the ozone generator is temporarily kept in a chamber (30 L working volume), before being pumped to the reactor (30 L working volume). Both tanks (chamber and reactor) are made of stainless steel 316. Two pistons (150 ml working volume) were especially constructed

using stainless steel 316 and silicone fittings for supplying ozone and applying vacuum to the reactor. Both pistons are driven by 0.5 hp motors (Bonfigliori Riduttori Spa, Italy) via two fully programmable inverters (type Altivar 11, Telemecanique). The reactor was kept at constant temperature and pressure using one Pt100 electrode and a Shimaden SR91 PID temperature controller, as well as a 627 type Capacitance Manometer and a 4000 Pressure readout unit (MKS, USA) respectively. Ozone measurement and control was achieved via an Ozomat GM Ozone Analyser and an Ozone Gas Cooler (ECP 1000) (ANSEROS GmbH). Humidity was measured using the wet/dry bulb method and a SHIM T30 humidity converter (Thema Automation, Greece). Steam produced using an ultrasonic humidifier was temporarily kept in a steam tank (30 L, SS 316) before being supplied to the reactor. Monitoring, operation and control of the system including control of ozone concentration, temperature, humidity and pressure was realized through a PCI-6025E I/O board and LABVIEW 7 (National Instruments) via a SCADA environment tailor-made to our application.

pH measurements

The pH measurement was carried out using an appropriate glass electrode (Electrode INLAB 420 of the company Mettler Toledo). In order to achieve precise and reliable measurements, three grapes of each cluster were tested every time.

Moisture content measurements

Table grapes were dried at 105 °C for 2 h (constant weight). The weight of the grapes was measured before and after drying.

Ozone measurements

Ozone measurements in gaseous phase were achieved via an Ozomat GM Ozone Analyser and an Ozone Gas Cooler (ECP 1000) (ANSEROS GmbH).

Furthermore the disinfectant factor is determined as the time integral of the ozone concentration in reactor and is given by the following formula:

$$c \times t = \int_t C_{O_3}(t) dt \quad (1)$$

where $C_{O_3}(t)$ is the ozone concentration as a function of time.

Visual observation-decay frequency (%)

The decay frequency (F%) was determined by dividing the number of diseased single berries (n) by the total number of berries (N) from each cluster of table grapes. As a result, the decay frequency (%) is given by the following formula:

$$F\% = (n/N) \times 100 \quad (2)$$

Weight loss

Weight loss (WL %) was calculated upon the following formula:

$$WL\% = [(W(t=0) - W(t))/W(t=0)] \times 100 \quad (3)$$

where $W(t=0)$ was the initial weight of each cluster of table grapes and $W(t)$ was their weight after days of ozone exposure.

The weight loss of table grapes was measured during their exposure to ozone atmosphere in order to study the effect of ozonation on physiological response of table grapes.

Carbohydrates measurements (Total and soluble)

The carbohydrate content of table grapes was based on the colorimetric method of Josefsson (1983). The soluble part (carbohydrates) was measured after the pulping of table grapes and filtering of the obtained liquid mixture, realized the suitable dilutions (1:10,000). The total part (sugars) was measured only after the pulping of the table grapes and realized the suitable dilutions (1:10,000).

Protein measurements

The protein content measurement of table grapes was carried out according to the Bradford protein assay (Bradford 1976). The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 to 595 nm when binding to proteins occur. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change.

Artificial microbial contamination techniques

Injection technique

For the evaluation of *B. Cinerea* nesting ability, 10 µL of a suspension of 10^6 spores mL⁻¹ of *B. Cinerea* were injected in the flesh of one central single berry per cluster using a Hamilton syringe (Hamilton Co., Reno, NV, USA). After the injection procedure, the table grapes remained in the

laminar flow hood, in order to recover their initial humidity.

Immersion technique

For the evaluation of gray mold incidence on grapes, a conidial suspension containing 10^6 spores·mL⁻¹ of *B. Cinerea* was uniformly immersed for about 5 s on clusters of ‘Sultanina’ table grapes. After the injection procedure, the table grapes remained in the laminar flow hood, in order to recover their initial humidity.

Preservation processes (low ozonation, high ozonation, periodical ozonation, initial ozonation)

In each storage and treatment process, there were two 2-cluster replicates for the immersion inoculation method and two 2-cluster replicates for the syringe inoculation method. The one 2-cluster replication of them was used for visual observation and the other one for the chemical analyses of table grapes. The number of infected grapes per cluster and the number of infected grapes surrounding the inoculated grape were recorded every 3 days.

Four different ozonation ways were developed and evaluated in order to study the impact of ozone on the decay development on table grapes caused by *B. Cinerea*. These ways were the following:

According to the low ozonation procedure, the table grapes were exposed in ozone atmosphere (ozonation time 60 min and average ozone concentration = 15 ppm, $c \times t = 900$ ppm min on a daily basis) and were stored for 40 days at 2–3 °C and 85–90% MC. Regarding to the high ozonation procedure, the table grapes were also exposed in an ozone atmosphere on a daily basis and were stored for 40 days at 2–3 °C and 85–90% MC. The ozonation time was 60 min but the ozone concentration was now higher and specifically 2500 ppm ($c \times t = 150,000$ ppm min on a daily basis). The table grapes according to the periodical ozonation procedure were exposed in an ozone atmosphere (ozonation time 60 min and high ozone concentration 2500 ppm) on a 3 day basis and were stored for 40 days at 2–3 °C and 85–90% MC ($c \times t = 50,000$ ppm min on a daily basis). According to the initial ozonation procedure, the table grapes were exposed only once in ozone atmosphere (ozonation time 60 min and high ozone concentration 2500 ppm) and were stored for 40 days and in the same temperature and humidity conditions as mentioned previously.

Last but not least, 2-clusters of table grapes were stored for 40 days at 2–3 °C and 85–90% MC, without any ozone treatment (control or blank experiment). The one cluster was also used here for visual observation and the other one for chemical analyses. The blank experiment was achieved

in order to compare all the measurements with those of the four procedures.

Results and discussion

Ozone treatment can be an alternative method in order to be controlled postharvest gray mold onto table grapes (Romanazzi et al. 2012). The caused decay by moulds in the grapes can be decreased with ozonation of the fruits (O'Donnell et al. 2012) and also, the postharvest ozone treatment can enhance the synthesis of resveratrol and other bioactive phenolics in grapes [as proved in works (González-Barrio et al. 2006; Cayuela et al. 2009)]. It is known that ozone and ozone-derived oxyradicals may be scavenged by low-molecular antioxidants in the plant cell (Schraudner et al. 1992) such as phenolic compounds.

One very important work was the one of Sarig et al. in which ozone treatment was used on peach fruit and grapes. It was concluded that an 80-min treatment of 4000 µl/l ozone had no effect on the firmness of grapes (Sarig et al. 1996). On the other hand, a 4000 µl/l ozone exposure for more than 40 min resulted in the degradation of the cuticle seen as microscopic veinlike cracks on the epidermis. The above ozone exposure induced the production of the stilbenes, resveratrol, and pterostilbene, and concentrations of these compounds peak 24 h after a five-to-ten-minute ozone treatment (Sarig et al. 1996). In addition at high concentration of O₃ (2 ppm) the levels of antioxidant compounds (e.g. resveratrol) produced by the plant can be decreased as a defensive metabolite against oxidative stress (Cayuela et al. 2009) ozone and ozone-derived oxyradicals may be scavenged by low-molecular antioxidants in the plant cell (Schraudner et al. 1992) such as phenolic compounds. In addition, at high concentrations of O₃ (2 ppm) the levels of antioxidant compounds (e.g. resveratrol) produced by the plant can be decreased as a defensive metabolite against oxidative stress (Cayuela et al. 2009).

Analysis of the four ozonation ways

1st experiment

The first experiment was carried out applying the low ozonation process combined with cold storage in order to study the effect of this treatment on the preservation time extension of table grapes. During this experiment, five 2-clusters of table grapes were used and several parameters, such as decay frequency (DF, %), weight loss (%), pH, moisture content (MC, %), sugars and proteins content were measured. The respective results are presented on the following Fig. 1a, b and in Table 1.

Fig. 1 Results about low ozonation process combined with cold storage for the preservation of 'Sultanina' table grapes: **a** weight loss (%), **b** decay frequency, DF (%)

According to the obtained results, all the table grapes clusters lost weight after their cold storage for 40 days and their treatment under low ozonation conditions or not. However, in the case of the ozonation exposure, the weight loss was significantly higher, especially in the case of the cluster which was artificially contaminated with *B. Cinerea* using the immersion technique. The same results were

observed also for the contaminated cluster via the injection process, but they were more obvious after the 20th day.

The incidence of gray mould caused by *B. Cinerea* was negligible for the treated clusters with ozone at low concentrations. The observed decay frequency levels for these clusters were similar with the observed decay for the control cluster. At the same time, the caused decay was extremely higher for the non ozonated clusters (60% for the contaminated cluster via immersion and 20% for the contaminated cluster via injection). Based on these results, it can be concluded that the developed process of low ozonation combined with cold storage had positive effect on the preservation time of table grapes.

After the processing of the table grapes, no remarkable changes were observed on the moisture content levels. In addition pH values were slightly lower comparing the starting value for all the clusters. Furthermore, no meaningful alterations on the sugars and proteins content were detected. This means that the processing didn't probably affect the quality parameters of table grapes.

Bibliographically (Smilanick et al. 1999), it is known that at 10 and 60 ppm min ozone treatment a reduction of gray mold from 35% among untreated grapes to about 10% was achieved.

2nd experiment

The second experiment was carried out applying the high ozonation process in order to study the effect of this treatment on the extension of the preservation time of table grapes. During this experiment, five 2-clusters of table grapes were used and several parameters, such as decay frequency (DF, %), weight loss (%), pH, moisture content (MC, %), sugars and proteins content were measured. The respective results are presented on the following Figure 1S (a and b) and in Table 1S of the Supporting Information.

The obtained results indicated that the ozonated samples lost significant weight and the weight loss was the same order of magnitude between the contaminated cluster via

Table 1 Quality parameters of 'Sultanina' table grapes before and after the application of low ozonation treatment combined with cold storage

Low ozonation								
Cluster	pH		MC (%)		Sugars (mg/kg)		Proteins (mg/kg)	
	Initial	End	Initial	End	Initial	End	Initial	End
Control	4.70 ± 0.09	3.96 ± 0.12	81.48	79.77	173.03 ± 1.57	184.10 ± 2.57	63.70	84.59
Bot. Imm. no O ₃	4.70 ± 0.09	3.96 ± 0.12	81.48	82.33	173.03 ± 1.57	142.50 ± 3.13	63.70	41.18
Bot. Inj. no O ₃	4.70 ± 0.09	4.08 ± 0.32	81.48	83.63	173.03 ± 1.57	140.76 ± 0.78	63.70	42.22
Bot. Imm. O ₃	4.70 ± 0.09	4.06 ± 0.22	81.48	85.23	173.03 ± 1.57	166.22 ± 4.47	63.70	81.92
Bot. Inj. O ₃	4.70 ± 0.09	3.98 ± 0.19	81.48	84.82	173.03 ± 1.57	179.51 ± 4.92	63.70	87.85

immersion and the other one via injection technique. The rest clusters were presented inconsiderable weight loss, but at similar levels comparing the achieved ones by the low ozonation process.

The incidence of grey mould caused by *B. Cinerea* was qualitatively similar for all the treated clusters with ozone at high concentrations. In another work (Feliziani et al. 2014) $c \times t$ values higher than ours (~ 3000 and 9000) were used. It was found that the natural incidence of gray mold was reduced by 65% after 5–8 weeks of storage but the decay by other fungi, such as *Alternaria* spp. and *Penicillium* spp. could not control by ozone. Also, in Gabler et al. work a significantly higher ozone concentrations (up to 10,000) have been used with similar results (Gabler et al. 2010).

In our case the decay frequency levels for the clusters treated with ozone were lower than the corresponding observed decay for the control cluster. In addition, the caused decay was sufficiently higher for the non ozonated clusters (100% for the contaminated cluster via immersion and 55% for the contaminated cluster via injection). Taking into account these results, it seems that the developed process of high ozonation combined with cold storage had also a positive effect on the preservation time of table grapes.

After the processing of the table grapes, the moisture content levels remained stable. Furthermore, pH values were slightly lower comparing the starting value for all the clusters. No major changes were remarked for the sugars and proteins content of table grapes. Consequently, it is concluded that the processing didn't probably degrade the quality parameters of table grapes.

3rd experiment

The third experiment was carried out applying the initial ozonation process for the same reason that has been mentioned to the previous experiments. During this experiment, five 2-clusters of table grapes were used and the parameters DF(%), weight loss (%), pH, MC (%), sugars and proteins content were measured. The respective results are presented on the following Figure 2S (a and b) and in Table 2S of the Supporting Information.

The application of the initial ozonation treatment combined with cold storage resulted to similar weight loss levels for all clusters of the table grapes. The control cluster lost a little less weight after 40 days storage, than the other clusters. The weight loss was limited up to 10% for the contaminated clusters (through immersion and injection technique), two-fold higher than the case of the control cluster.

No significant differences for the incidence of the gray mould were observed between the contaminated clusters

either treated or not, with initial ozone dosage. Decay on control cluster was retained at low levels. The decay caused by *B. Cinerea* on the table grapes was visible, as long as the decay frequency reached 100% for the contaminated clusters. The contaminated cluster using the immersion technique, treated with ozone (Bot. Imm. Initial O_3) was the exception thus the decay frequency on it, reached 50%. This fact probably is attributed not to the ozone effect, but to other factors such as the grape density of each cluster. Finally, it seems that the initial use of ozone did not affect the rate of decay development on the table grapes.

After the processing of the table grapes, the moisture content levels and pH values remained stable for all the clusters. No significant changes were observed for the sugars and proteins content of table grapes. Based on these, it is concluded that the processing didn't probably modify the quality parameters of table grapes.

4th experiment

The fourth experiment was carried out applying the periodical ozonation process in order to study the effect of this treatment on the extension of the preservation time of table grapes. During this experiment, five 2-clusters of table grapes were used and several parameters, such as decay frequency (DF, %), weight loss (%), pH, moisture content (MC, %), sugars and proteins content were measured. The respective results are presented on the following Figure 3S (a and b) and in Table 3S of Supporting Information.

The contaminated clusters treated with ozone periodically, lost higher weight comparing the untreated contaminated clusters. This was cleared after the first 15 days of the treatment. The control cluster lost a little less weight after 40 days storage, than the other clusters. The weight loss for the contaminated clusters treated with ozone approached 15–20%, two-fold higher than the case of the untreated contaminated clusters.

No remarkable differences for the incidence of the gray mould were observed between the contaminated clusters treated or no with periodical ozone dosage. Decay on control cluster was retained at low levels. The decay caused by *B. Cinerea* on the table grapes was visible, as long as the decay frequency reached 100% for the contaminated clusters. Consequently, the periodical ozonation hadn't any inhibited result on decay development on the table grapes.

The processing of the table grapes didn't change the moisture content levels, pH values and sugars, proteins contents which remained stable for all the clusters. Upon these, it is concluded that the processing didn't probably modify the quality parameters of table grapes.

Comparison

The current work constitutes a complete study of different ozonation ways of table grapes in order to extend their shelf-time under cold storage. The most referred ozonation way, cited in the literature (Palou et al. 2002) includes the continuous exposure of table grapes and other fruits in an ozone atmosphere. According to the current study, novel ozonation processes are introduced. These treatments include the application of a specific low or high ozone dosage for 1 hour every day, for 1 hour at the starting of the storage time and for 1 hour every 3 days. The realization of these processes was carried out in order to find the optimum ozone dosage either of technical or economical point of view for improvement the storage conditions of table grapes. According to the obtained results it is obvious that the exposure of table grapes to ozone atmosphere resulted to significant weight losses, higher than the respective obtained by the control or untreated clusters. This ozone effect to the physiological response of table grapes was more intent at the application of high ozonation and periodical ozonation processes (up to 20%). The incidence of the gray mould caused by *B. Cinerea*, was restricted after the application of the low and high ozonation processes. The initial and periodical ozonation was proved to be insufficient for prevention the decay development, as long as the decay frequency reached 100% in almost of the cases. It seems that the daily ozone treatment is necessary in order to secure the preservation of table grapes. In addition, the measurements about the quality parameters of table grapes during their storage time (data shown only for the start and the end point) indicated that the ozonation did not significantly alter their levels in table grapes. The last conclusion is coming to consolidate the FDA approval for the use of ozone in food industry and make clear that the ozone is a powerful disinfectant agent with multiple uses. As a result, the suggested process for table grapes conservation, taking into account the achieved results and the financial cost for ozone generation, is the low ozonation process. In addition the applied ozone dosage according to the developed process is close to the 0.3 ppm, which is the Threshold Limit Value (TLV) established by the United States Occupational Safety and Health Administration (US-OSHA). Thus the cost for aeration of the warehouse used for table grapes preservation is significantly reduced. Consequently, the low ozonation process combined with cold storage is the optimum treatment from technical and economical point of view for conservation of ‘Sultanina’ table grapes.

Conclusion

Ozone has many uses and applications in human civilization. One of the most interesting application of ozone is located at the storage, treatment and processing of foods after the recent approval by the U. S. Food and Drug Administration. In the current study, alternative ozonation processes for table grapes preservation were developed and evaluated, in order to choose the optimum from technico-economical point of view. The ozone treatment of table grapes on a daily basis with ozone dosage $c \times t = 900$ ppm min, combined with cold storage (2–3 °C, 85–90% MC) proved to be the most efficient preservation process. The results indicated that ozone inhibited the decay development caused by *B. Cinerea* on the fruit. The observed weight loss remained at low levels and no sugars or proteins alteration was observed. Consequently, the suggested process can be tested on different kinds of fruits contaminated with various types of moulds.

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