Table 1. Characterization of fruiting bodies produced by protoclones of *Pleurotus ostreatus* (ATCC60691).

Strain	Cultivation[z] period (days)	Stipe diam (mm)	Pileus diam (mm)	Fruiting bodies No.[y]	Fruiting bodies Weight (g)
P1[x]	11	7.0	27.3	14.3	66.3 ± 9.1
P2[x]	8	7.2	25.7	13.3	68.7 ± 5.0
1	6	6.8	26.3	15.2	75.5 ± 3.7 *,**
5	5	7.3	26.5	14.3	71.8 ± 7.9 *
17	6	7.2	24.5	15.2	75.5 ± 2.9 *,**
19	5	7.7	25.7	15.0	74.3 ± 1.5 *
20	6	7.3	27.0	14.2	73.3 ± 3.7 *
29	6	6.7	22.0	14.0	59.8 ± 7.7 **

[z]Period taken for primordium to be formed after the low temperature treatment.
[y]Number of fruiting bodies with pileus larger than 20 mm in diameter.
[x]P1 = Parent strain maintained on potato dextrose agar slant. P2 = Parent strain grown in liquid medium and used for protoplast isolation. Significant difference at 10% level from P1(*) or P2(**); LSD = 6.7. Results are the mean ± SD of six replicates.

Table 2. Poststorage characteristics of fruiting bodies produced by strains that showed significant yield increase before their storage at 2°C for 138 days.

Strain	Cultivation period (days)	Fruiting bodies No.	Fruiting bodies Wt (g)
P2	12	13.0	58.2 ± 6.2
1	11	17.6	55.6 ± 5.1
17	12	18.9	60.7 ± 7.0
19	12	14.9	60.0 ± 7.2

the protoclones was the shorter time period taken for primordia formation after the induction treatment (Fig. 1). Fewer than 6 days were needed for primodia of the protoclones to appear, whereas, 8 days for P2, and 11 days for P1 (Table 1). This was a common feature observed in all the protoclones.

Three of the protoclones (1) that showed a significant increase in yield of fruiting body and P2 were re-evaluated in a second trial. They had been kept as sawdust spawn at 2°C for 138 days before the examination. The main feature of the protoclones, greater yield in shorter period, was lost after having been maintained at 2°, when all yields were similar (Table 2).

In the first experiment of this work, the increase in fruiting body yield of five out of 29 protoclones was significant. But the second experiment showed that the capability of producing a yield higher than the parent was lost. Apparently, the physiological state induced by protoplasting and regeneration could not be maintained during storage at 2°C for 138 days. The factors that cause the faster growth and increase in fruiting body weight are not known. The same type of phenomena are reported with secondary metabolite-producing cell lines, such as in *Catharanthus roses* (9). Isolated variants of *C. roses* rapidly lost their capacity for high production of indole alkaloids during propagation.

In conclusion, although six oyster mushroom protoclones were obtained among 29 with statisticly significant increases in yield, they were found not to be stable.

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HortScience 23(6):1066–1068. 1988.

Guidelines for Autoclaving Liquid Media Used in Plant Tissue Culture

David W. Burger
Department of Environmental Horticulture, University of California, Davis, CA 95616

Abstract. **The temperatures of liquid volumes (10–4000 ml) were monitored with solid-state temperature probes during autoclaving cycles of an automated steam sterilizer. Accurate measurements of the time required for the liquid volumes to reach the sterilizing temperature (121°C) were made. The effects of preheating solutions to be autoclaved and enclosing materials in autoclavable plastic bags were determined. Liquid volumes between 10 and 4000 ml differed in the time required to reach 121° by 40 min. Preheating solutions before autoclaving slightly reduced the time required to reach sterilizing temperatures. Enclosing materials in autoclavable bags substantially increased the time required to reach 121°.**

The use of autoclaves to sterilize equipment and media for in vitro culture studies is widely practiced. It is generally recommended that a solution must reach and maintain a temperature of 121°C for 15 min to ensure sterility (Howie, 1959). Our studies involving large volumes (2–4 liter) of liquid media have indicated that the commonly recommended exposure times (Biondi and Thorpe, 1981; Meynell and Meynell, 1970) are insufficient. There is a lack of published experimentation that directly answers the question of how long to autoclave given volumes of liquid to ensure sterility. In general practice, liquids are autoclaved for the designated length of time, usually overestimated, to ensure sterility; thus, increasing the probability of overexposing heat labile compounds.

Biondi and Thorpe (1981) have suggested that as the volume of the liquid increases so does the necessary minimum sterilization time; however, supporting details were not provided. Beverloo (1971) monitored the temperature of 0.5 to 1.0 liter cans distributed throughout the autoclave and found that temperature depended on location. Gillespie and Gibbons (1975) monitored autoclave cycles with thermocouples to determine whether there were differences in the internal location and the temperatures that were achieved relative to the type of support used. Review articles (Meynell and Meynell, 1970), research publications (Perkins, 1969), and committee reports (Howie, 1959) have been concerned with the uniformity and efficacy of autoclaves used for sterilization. Perkins (1969) did not observe the temperature of

Received for publication 25 Jan. 1988. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

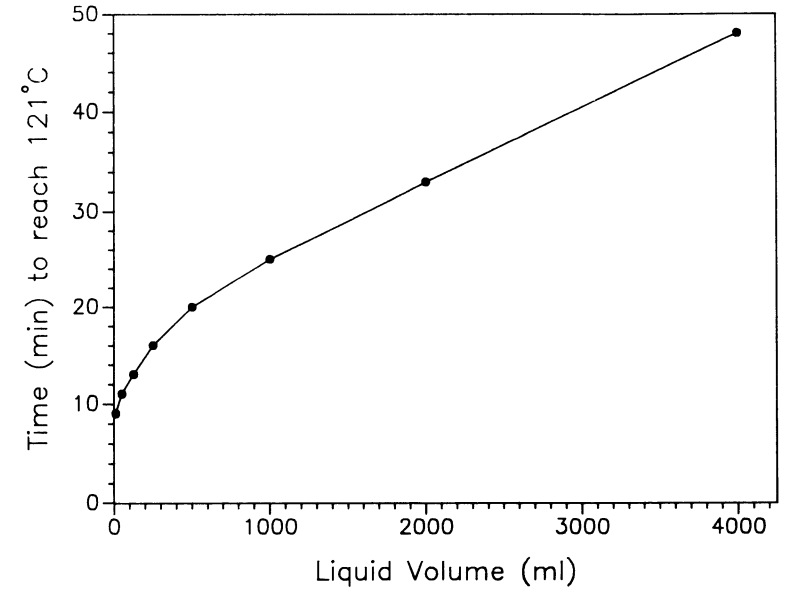

Fig. 1. Required time (min) for liquid volumes (10–4000 ml) to reach 121°C.

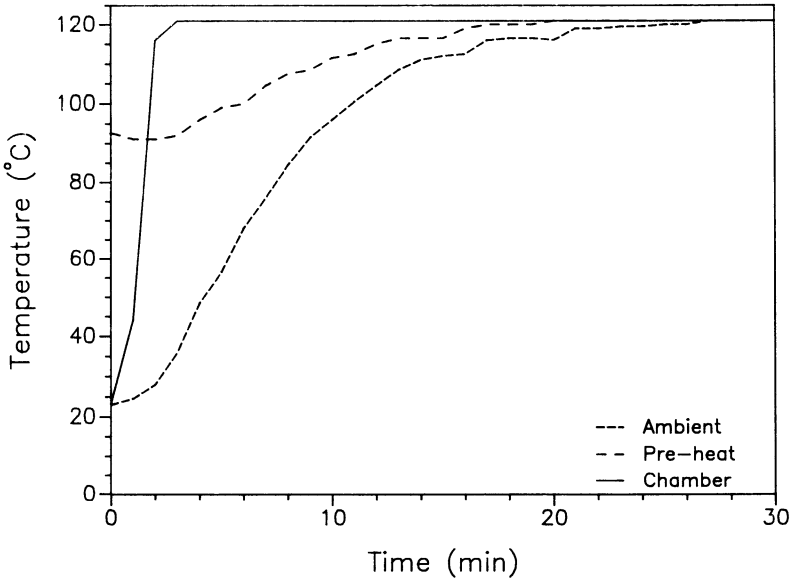

Fig. 2. Effect of initial liquid temperatures (ambient, preheated) on the time required to reach 121°C.

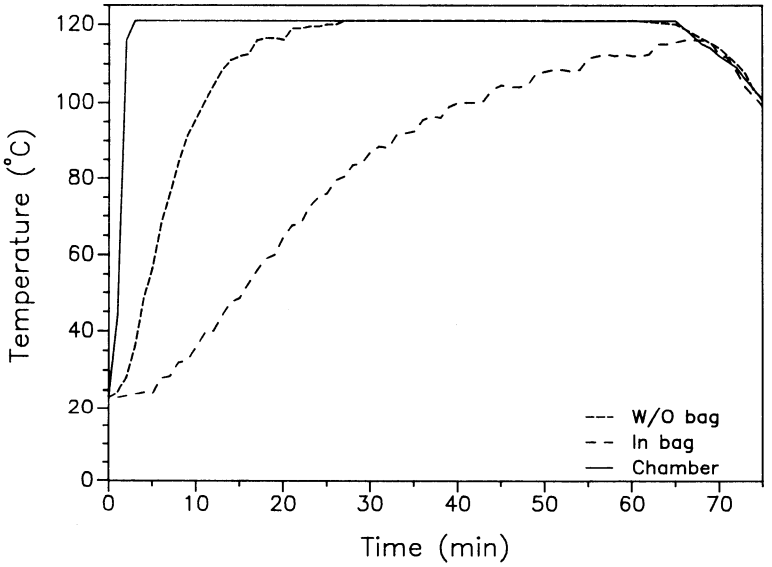

Fig. 3. Effect of wrapping flasks containing liquid volumes in autoclavable bags on the time required to reach 121°C.

single and small groups of vessels in the autoclave; he filled the autoclave chamber with many samples of the same volume and noted temperature changes during the autoclave cycle. In a committee report (Howie, 1959), the time individual volumes of liquid required to reach 115°C, 6° less than the sterilizing temperature, was cited from a personal communication.

Sterilization process indicators (i.e. Diack control) may be used to indicate whether a critical temperature has been reached, but do not indicate the length of time the critical temperature was maintained. The literature has not addressed the central question most plant tissue culture researchers ask: What is the minimum autoclave cycle time required to ensure that the entire liquid volume has been sterilized? This question is most important when large volumes of liquid (media or rinse water) are autoclaved and is the question addressed in this report.

An automated, steam autoclave (AMSCO model LB-15AS, Electric Steam Generator) was used for all sterilizing cycles. Solid-state temperature probes designed to withstand high temperature and pressure were placed both in the vessels to be autoclaved and suspended in the chamber. The output end of the probes was connected to an A/D (analog/digital) converter (OWL87, EME Systems, Berkeley, Calif.), which was attached to a portable computer (Radio Shack, Model 100). This data logging system continuously monitored up to six probes during each autoclaving cycle. Wires connecting the sensors to the A/D converter were taped to the opening of the autoclave with autoclave tape to prevent pressure leakage during the cycle and to protect them from the extreme heat generated by the autoclave jacket.

Expt. 1 involved 25- to 2000-ml glass Erlenmeyer flasks containing 10 to 1500 ml of deionized water, respectively, and a 4-liter glass, aspirator bottle containing 4 liters of deionized water that were placed into the autoclave alone or in pairs with one temperature probe suspended in the middle of each liquid volume. The liquid volume was 75% of the overall volume of the flasks (i.e. 750 ml in a 1000-ml flask). An additional autoclave test for surviving contaminants was conducted using a nutrient-broth culture medium. The culture medium consisted of 8 g of Difco-Bacto Nutrient broth and 20 g of sucrose per liter of medium (pH 6.5 before autoclaving). A nutrient-broth medium was chosen to favor the growth of any bacteria or fungi that might be viable after the autoclaving treatments. Culture medium, 25 or 50 ml, was distributed into 125-ml glass culture flasks and autoclaved for 1, 2, 5, 10, 15, or 20 min (three replicate flasks of each volume for each time period). The flasks were fitted with polypropylene slip-top caps. After autoclaving, the flasks were allowed to cool in a laminar flow hood. Upon cooling, the junction between the cap and the neck of the flask was wrapped with Parafilm and placed on a horizontal shaker (26°–28°C). After 1 week the incidence of contamination (bacteria, fungi) was noted by visual (cloudiness

of the solutions) and microscopic examination.

In a second experiment, a 1000-ml glass Erlenmeyer flask containing 750 ml of water was preheated to 90° to 95°C in a microwave oven before being placed into the autoclave with a 1000-ml flask containing 750 ml of water at room temperature (25°). Lastly, a 2000-ml flask containing 1500 ml of water was fitted with a temperature probe, completely enclosed in an autoclavable plastic bag and placed along side a 2000-ml flask containing 1500 ml of water without enclosure in a bag. Autoclave cycles were not initiated until the jacket pressure reached 1.4 $kg \cdot cm^{-2}$. In each run, the chamber reached 121° within 2 to 3 min. All autoclave cycles were run at least twice.

As the liquid volume in the flask increased, the time needed to reach 121°C also increased (Fig. 1). Given that 15 min at 121° is considered necessary to sterilize liquids (Howie, 1959), the total time necessary to autoclave a certain volume will be the time necessary to reach 121° plus 15 min. For example, it took 1000 ml ≈25 min to reach 121°, which should be maintained for 15 min. Thus, the total time necessary to sterilize completely 1000 ml of liquid is about 40 min. Recommendations in the literature usually suggested 20 to 30 min for this volume (Biondi and Thorpe, 1981; Perkins, 1969).

Flasks containing 25 to 50 ml of nutrient broth required at least 20 min of autoclaving to remain absolutely sterile. Only the flasks containing 25 ml of medium autoclaved for 20 min were 100% sterile. None of the flasks containing 25 ml of medium autoclaved for <10 min were sterile. Among flasks containing 25 ml of medium autoclaved for 10 to 15 min, 67% and 33% were contaminated, respectively. The contamination was due to the presence of bacteria and fungi. Among flasks containing 50 ml, 100%, 100%, 100%, 67%, 67%, and 33% were contaminated after autoclaving for 1, 2, 5, 10, 15, and 20 min, respectively.

Preheating 750 ml of water in a 1000-ml flask to 90°–95°C before autoclaving reduced the time necessary to reach 121° from 26 to ≈20 min (Fig. 2). Preheating solutions before autoclaving may be an efficient way of reducing the overall time that liquids are exposed to autoclave conditions and is routinely accomplished during procedures to melt agars.

Enclosing a 2000-ml glass Erlenmeyer flask containing 1500 ml of water in an autoclavable bag substantially increased the time needed to reach 121°C (Fig. 3). In fact, after 65 min of exposure to 121° and 1.05 $kg \cdot cm^{-2}$ pressure, the flask in the bag had not yet reached 121° whereas, the uncovered flask had reached that temperature in 35 min. After 65 min the exhaust portion of the cycle began. This result emphasizes the need to remove all air from the internal chamber of the autoclave so that steam can be exposed to all materials in the autoclave. The retention of air in autoclave chambers has been noted as one of the common faults of inefficient sterilization practices (Howie, 1959).

With the data presented here, one may determine the length of time required for the volume of liquid in question to reach sterilizing temperatures and accurately set the autoclave to expose the volume to that time plus 15 min. Preheating liquids before autoclaving can reduce the time necessary to expose liquids to harsh, autoclaving conditions. This may be helpful when autoclaving solutions containing substances that are somewhat heat labile. The practice of enclosing materials in sealed, autoclavable bags should be avoided.

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HortScience 23(6):1068–1070. 1988.

Induction of Morphogenic Callus Cultures from Leaf Tissue of Garlic

Akitsu Nagasawa[1] and John J. Finer

Department of Agronomy, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, OH 44691

Additional index words. *Allium sativum*, tissue culture, auxin, regeneration

Abstract. **Morphogenically regenerable callus was induced from young leaf and meristem tissues of garlic (*Allium sativum* L. cv. Howaito-Roppen). Five auxins were compared for their ability to induce morphogenic callus. In order of decreasing effectiveness, 2,4-D (0.1–3.0 $mg \cdot liter^{-1}$), 2,4,5-T (0.3–10 $mg \cdot liter^{-1}$), dicamba (10–30 $mg \cdot liter^{-1}$), and picloram (10–30 $mg \cdot liter^{-1}$) were capable of morphogenic callus induction, while NAA did not induce morphogenic callus formation over a wide range of concentrations. The morphogenic callus was nodular and gave rise to plantlets following transfer to medium containing BA. Chemical names used: (2,4-dichlorophenoxy)acetic acid (2,4-D); (2,4,5-trichlorophenoxy)acetic acid (2,4,5-T); 3,6-dichloro-2-methoxybenzoic acid (dicamba); 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid (picloram); 1-naphthaleneacetic acid (NAA); and *N*-(phenylmethyl)-1*H*-purin-6-amine (BA).**

Improvement of garlic (*Allium sativum* L.) through classical breeding techniques is not possible because cultivated garlic is sexually sterile. Standard vegetative reproduction of this crop has resulted in low propagation rates and the transmission of virus diseases. For this reason, in vitro techniques have been developed for garlic. Callus culture and plant regeneration (1, 5, 7), stem tip culture (9), shoot proliferation (2), and cold preservation of important germplasm (3) have been reported. In spite of the numerous publications on tissue culture of garlic, basic protocols have been rather limited. Previous reports used media containing a mixture of growth regulators (auxin and cytokinin) and stem tips as explant sources. This report demonstrates that young leaf tissue can also be a source of regenerable callus. The use of leaf tissue as a explant source is desirable because many explants can be derived from a single shoot, whereas only one stem tip can be obtained from a single shoot or clove. Regenerable callus was induced by a single addition of several synthetic auxins, some of which have never been evaluated in a garlic tissue culture system.

In vitro plantlets regenerated from a proliferating shoot culture of a commercial Japanese garlic, 'Howaito-Roppen' (six white cloves), were used as the explant source in this study. Initially, stem tip-derived callus was initiated and maintained on a shoot proliferation medium [modified from Oosawa et al. (8)] containing Murashige and Skoog (MS) salts (6), Gamborg's B5 vitamins (4), 1 mg NAA/liter, 2 mg BA/liter, 30 g sucrose/liter

Received for publication 25 Jan. 1988. Salaries and research support were provided by Sumitomo Chemical Co., Ltd. and by State and Federal funds appropriated to OSU-OARDC. OARDC Journal Article no. 18-88. We wish to thank Barbara Norris and Martha Fockler for their technical assistance. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

[1]Visiting Scientist, present address: Sumitomo Chemical Co., Ltd., Biotechnology Laboratory, Takarazuka Research Center, 4-2-1 Takatsukasa, Takarazuka, Hyogo 665, Japan.