1990

Stability of IAA and IBA in Nutrient Medium to Several Tissue Culture Procedures

Scott Nissen
University of California - Davis

Ellen Sutter
University of California - Davis

HORTSCIENCE 25(7):800-802. 1990.

Stability of IAA and IBA in Nutrient Medium to Several Tissue Culture Procedures

Scott J. Nissen[1] and Ellen G. Sutter
Department of Pomology, University of California, Davis, CA 95616

Additional index words. biotechnology, plant growth regulators

Abstract. **The relative stabilities of IAA and IBA under various tissue culture procedures were determined. IBA was significantly more stable than IAA to autoclaving. IBA was also found to be more stable than IAA in liquid Murashige and Skoog medium (MS) under growth chamber conditions. The stabilities of IBA and IAA were similar in agar-solidified MS. Light provided by cool-white fluorescent bulbs promoted degradation of IAA and IBA in both liquid and agar media. Activated charcoal in concentrations as high as 5% was found to adsorb more than 97% of IAA and IBA in liquid MS. These results have important implications for the preparation, storage, and handling of IBA and IAA in plant tissue culture. Chemical names used: indole-3-acetic acid (IAA); indole-3-butyric acid (IBA).**

Natural and synthetic auxins have been used extensively in plant cell, tissue, and organ cultures to elicit specific morphogenetic responses (Bhojwani and Razdan, 1983). It is well known, however, that results using similar protocols, types and concentrations of auxins may differ significantly among laboratories, hindering development of standard protocols in fields such as micropropagation. In particular, diverse handling procedures for plant growth regulators are a potential source of variability. Despite widespread use of these compounds, limited information is available on their stability under tissue culture conditions.

IAA continues to be used in plant tissue cultures (Dai et al., 1987; Grout and Read, 1986; Srivastava et al., 1985) in spite of evidence suggesting that it is unstable. The use of auxins, such as IBA, naphthaleneacetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D), has increased because they often appear to be more effective than IAA for induction of morphogenetic responses. This enhanced effectiveness of auxins other than IAA is presumed to be due to their increased stability. IBA is preferred for adventitious root induction in apple and other woody species grown in vitro, since it is more effective than IAA (James, 1983). Dunlap et al. (1986) recently demonstrated that 2,4-D and NAA had greater long-term stability than IAA in liquid Murashige and Skoog medium. Phenylacetic acid (PAA) has also been shown to be relatively stable during in vitro culture (Leuba et al., 1989).

This study was undertaken to determine whether the relative effectiveness of IBA, compared to that of IAA, is due to increased stability under in vitro conditions. These two auxins were compared for relative stability to autoclaving and under growth chamber conditions when incorporated in media with and without activated charcoal.

IAA and IBA concentrations were determined by reverse phase HPLC coupled with fluorescence detection. The HPLC was an LDC Gradient Master with two Constametric II pumps that provided isocratic conditions of 60% water (EM Science, Gibbstown, N.J.) containing 0.1% HPLC-grade acetic acid (J.T. Baker, Phillipsburg, N.J.) and 40% acetonitrile (American Burdick and Jackson, Muskegon, Mich.). An Applied Biosystems (Foster City, Calif.) 980 fluorescence detector was set for an excitation wavelength of 220 nm, and the emitted light was monitored using a 350-nm band-pass filter. IAA and IBA had retention volumes of 3.2 and 6 ml, respectively, at an eluant flow rate of 1.5 $ml \cdot min^{-1}$. Auxin concentrations as low as 1 ng/20 µl could be measured with confidence; therefore, we could readily measure up to 97% reductions in the original 10-µM concentrations in media. Direct 20-µl injections of 10-µM IAA and IBA aqueous solutions contained 34.4 ± 1.5 and 39.0 ± 1.0 ng of IAA and IBA, respectively.

Liquid culture medium was injected directly into the HPLC. Auxins were extracted from agar-solidified medium using anhydrous ether. Before extraction, 10,000 dpm of ^{14}C-labeled IAA or 30,000 dpm of ^{3}H-labeled IBA was added to the 1-ml agar samples. The agar was slurried with 2 ml of 100 mM K_2HPO_4 buffer adjusted to pH 2.7 with H_3PO_4. Anhydrous ether (2 ml) was added, and the test tubes were vortexed for 2 min and the&centrifuged (2000 × *g)* to sediment the agar. A syringe was used to transfer 1 ml of the ether supernatant to 1.5-ml microcentrifuge tubes. The ether was evaporated to dryness under N_2 and samples were resuspended in 100 µl of methanol. The percent recovery was determined by collecting the IAA and IBA fractions and determining the amount of radioactivity using a Packard (Grove, 111.) TRI-CARB 2200CA liquid scintillation counter.

Factors studied for their effect on stability of IAA and IBA were: 1) the effect of autoclaving vs. filter sterilization of the auxins, 2) pH of the medium before autoclaving, 3) length of time of autoclave cycle, 4) light vs. dark during incubation of media in a growth chamber, 5) agar-solidified vs. liquid medium, and 6) the addition of activated charcoal to liquid medium. The standard medium (MS) used was Murashige and Skoog (1962) basal salts, vitamins, and sucrose. Each time medium was prepared, fresh stock solutions of auxins were made at 10-mM concentrations in 95% ethanol. Standard autoclave conditions were 20 min at 121C. IAA or IBA (Sigma, St. Louis) was added at 10 µM to all treatments, except as indicated in studies with charcoal. Each treatment combination described below was prepared in triplicate and each experiment was conducted twice. Data were analyzed using Student-Newman-Keuls multiple-stage test (Miller, 1981).

The effect of autoclaving and pH were studied simultaneously. MS media containing IAA or IBA were adjusted to pH 5.0 or 5.7 with KOH before being autoclaved; each set of media was then autoclaved for 20 to 60 min. A similar set of treatments at pH 5.0 and 5.7 was prepared, except that IAA and IBA were added by filter sterilization through a sterile 22-µ Acrodisc filter (Gelman, Ann Arbor, Mich.) after the autoclaved media had cooled to 60C. All media were handled as 250-ml aliquots in 500-ml screw-

Received for publication 28 Aug. 1989. 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]Present address: Dept. of Agronomy, Univ. of Nebraska, Lincoln, NE 68583-0915.

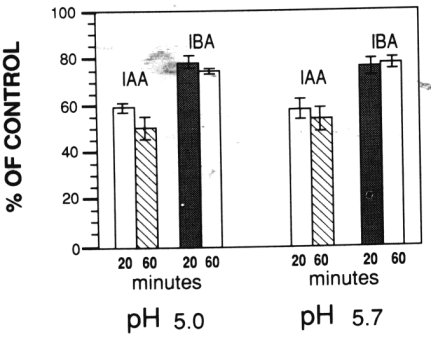

Fig. 1. The effect of autoclaving and pH on the stability of IAA and IBA in liquid MS medium. Means are expressed as the percentage of filter-sterilized controls. Vertical bars denote SE.

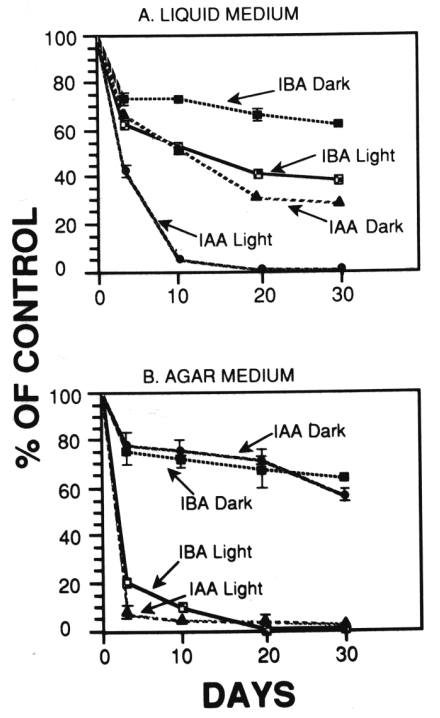

Fig. 2. Stabilities of IAA and IBA under in vitro conditions in liquid **(A)** and agar-solidified **(B)** MS media. The amount of hormone remaining is expressed as the percentage of the initial concentration of 10 µM. Vertical bars denote SE. Symbols without vertical bars have an SE smaller than the height of the symbol.

capped Erlenmeyer flasks. The effect of light on stability of IAA and IBA was determined in both liquid and agar-solidified MS. Liquid MS was prepared as 250-ml aliquots as described above. Difco bacto agar, at 0.8% (w/v), was added to 250-ml aliquots of liquid MS before being autoclaved. All media were adjusted to pH 5.7 before autoclaving. IAA or IBA was added by filter sterilization to autoclaved medium cooled to 60C. The liquid medium was then dispensed as 1-ml aliquots into sterile, screw-capped glass culture tubes (25 ml total volume capacity). The agar medium was dispensed as 10-ml aliquots into 60 × 15-mm glass petri dishes. One half of the tubes and petri dishes were wrapped in aluminum foil to exclude light. All vessels were incubated in a Percival growth chamber (Boone, Iowa) without shaking at 24 ± 2C, with a 16-hr photoperiod and a photosynthetic photon flux of 74 $\mu mol \cdot s^{-1} \cdot m^{-2}$ provided by cool-white fluorescent bulbs. Three tubes and three petri dishes were selected randomly from each treatment at each of 2, 10, 20, and 30 days and the medium was analyzed for IAA and IBA as described above.

The effect of activated charcoal (AC) (Mallinckrodt, St. Louis) was tested in liquid MS. Experiments were set up to test both the effects of adding various concentrations of AC to a constant auxin concentration and the effect of adding a single concentration of AC to a range of auxin concentrations. AC at 0.1%, 0.25%, 1.0%, and 5.0% (w/v) was added to 10 ml of liquid MS containing 10 µM IAA or IBA. The culture tubes were vortexed for 3 min and allowed to Stand in darkness for an additional 60 min. Centrifugation (2000× *g)* was used to sediment the suspended charcoal. The adsorptive capacity of charcoal was examined by adding 25 mg of AC to 10 ml of liquid MS containing 10, 50, 100, 500, or 1000 µM IAA or IBA. Test tubes were vortexed and charcoal was sedimented as described above.

The concentrations of IAA and IBA in autoclaved liquid MS were reduced by 40% and 20%, respectively, compared with filter-sterilized controls (Fig. 1). IBA was more stable than IAA in both autoclave conditions tested. Duration of autoclaving and pH of the medium did not affect IAA or IBA losses. Our results, indicating that losses of both auxins were substantial after autoclaving for 20 min, differ from earlier reports (Dunlap and Robacker, 1988; Pence and Caruso, 1984; Yamakawa et al., 1979). Pence and Caruso (1984) used a root initiation bioassay rather than direct measurement of IAA, a method that may have given spurious results. Yamakawa et al. (1979) determined IAA levels using the indole-a-pyrone method, a procedure that has found limited acceptance because of problems with interfering compounds (Crozier et al., 1980). We cannot explain the difference between our results and those of Dunlap et al. (1986), who found that IAA was stable to autoclaving.

Under growth chamber conditions, IAA and IBA losses from both liquid (Fig. 2A) and agar-solidified (Fig. 2B) MS were significant. In liquid medium, IAA was more sensitive than IBA to nonbiological degradation (Fig. 2A). In the light, IAA and IBA concentrations were reduced by more than 97% and 60%, respectively, after 20 days. In the dark, IAA and IBA concentrations declined by 70% and 30%, respectively. IAA losses from liquid MS were similar to those reported previously (Dunlap and Robacker, 1988; Dunlap et al., 1986; Yamakawa et al., 1979). Dunlap et al. (1986) reported that an increased concentration of MS salts correlated with an increased rate of IAA breakdown in the dark. Our finding that IAA deteriorates more in light than in darkness, regardless of the presence of agar, confirms a previously published study (Dunlap et al., 1986).

The losses of IAA and IBA in agar-solidified MS (Fig. 2B) were considerably different from those in' liquid MS. In darkness, in agar-solidified MS, IAA and IBA concentrations were reduced by 45% and 38%, respectively, after 30 days. After only 3 days in the light, however, the losses of IAA and IBA were »95% and 80%, respectively (Fig. 2B). Dunlap and Robacker (1988) suggested that salts and micro-nutrients in liquid MS interact to accelerate IAA breakdown in the light. Agar has also been shown to contain salts, micronutrients, and other unknown impurities (Debergh, 1983) that could interact with light to further accelerate the breakdown of IAA and IBA over that in liquid MS. We cannot offer an explanation for the »35% reduction of IBA in darkness without further analysis of metabolites of IBA. Additional studies analyzing breakdown products of IBA would be necessary to explain the degradative process that occurs in darkness.

Activated charcoal, added to liquid MS medium at concentrations ranging from 0.1% to 5% (w/v), reduced IAA and IBA concentrations by more than 97%. The addition of 0.25% AC to liquid MS containing 10 to 500 µM IAA or IBA adsorbed virtually all auxin At 1000 µM auxin, only 44.7% of IAA and 12.5% of IBA remained in solution. Previous investigators, using bioassays to quantify auxin concentrations, have suggests that activated charcoal adsorbs plant growth regulators in addition to autotoxic compounds (Fridborg and Eriksson, 1975; Fridborg et al., 1978). To our knowledge, our study is the first to directly determine auxin concentrations in liquid MS medium supplemented with AC.

The results of this study indicate that tissue culture procedures can significantly affect the stability and availability of IAA and IBA. The decrease in concentration of IAA and IBA, particularly in agar-solidified medium in the light, has important implications for the storage of media. It is clear that in as little as 10 days of storage, the majority of auxin could be degraded. Thus, the time of media preparation in relation to incubation of tissue samples is a potential source of variation among laboratories.

The use of auxin conjugates has been proposed as a possible substitution for IAA (Hangarter et al., 1980). Their use is limited however, since their effects were often not similar to those of IAA. For example, IAA-alanine, the most effective conjugate tested by Hangarter's group for callus growth, clearly inhibited root and shoot growth in marked contrast to the effect of IAA (Hangarter et al., 1980). In addition, although IAA conjugates are known to be more stable than IAA in plant tissues, no studies have been done that directly determined their stability to autoclaving, pH, light, and storage.

Our research did not attempt to explain the difference in effectiveness between IAA and IBA when used in vitro. The generally accepted view that the superiority of IBA over IAA in vitro is due to its relatively higher stability is supported by our tests. Apparently, IBA is significantly more stable than

IAA in liquid MS. In agar-solidified MS, however, the stability of IBA is similar to that of IAA, suggesting that other factors relating to the biology of IBA itself may contribute to the reported higher activities of IBA in vitro. Factors, such as differential metabolism of IAA and IBA, may contribute to the relatively greater effectiveness of IBA over that of IAA (James, 1983). It is also important to note that activated charcoal significantly reduced the availability of exogenous IAA and IBA at concentrations of auxin up to 500 µM. Thus, when activated charcoal is used to adsorb toxic compounds in tissue culture media, concentrations of IA4 and IBA might have to be increased 10 to 100 fold to ensure their availability.

Literature Cited

Bhojwani, S.S. and M.K. Razdan. 1983. Plant tissue culture: Theory and practice. Elsevier, Amsterdam, The Netherlands.

Crozier, A., K. Loferski, J.B. Zaerr, and R.O. Morris. 1980. Analysis of picogram quantities of indole-3-acetic acid by high performance liquid chromatography fluorescence procedures. Planta 150:366-370.

Dai, C., V.N. Lambeth, R. Raben, and D. Mertz. 1987. Micropropagation of *Rhododendron prinophyllum* by ovary culture. HortScience 22:491-493.

Debergh, P.C. 1983. Effects of agar brand and concentration on the tissue culture medium. Physiol. Plant. 59:270-276.

Dunlap, J. and K. Robacker. 1988. Light and nutrient salts interact to destroy IAA in tissue culture media. Plant Physiol. 86:120.

Dunlap, J.R., S. Kresovich, and R.E. McGee. 1986. The effect of salt concentration on auxin stability in culture media. Plant Physiol. 81:934-936.

Fridborg, G. and T. Eriksson. 1975. Effects of activated charcoal on growth and morphogenesis in cell cultures. Physiol. Plant. 34:306-308.

Fridborg, G., M. Pederson, L. Landstrom, and T. Eriksson. 1978. The effect of activated charcoal on tissue cultures: Adsorption of metabolites inhibiting morphogenesis. Physiol. Plant. 43:104-106.

Grout, J.M. and P.E. Read. 1986. Influence of stock plant propagation method on tissue culture and leaf-bud propagation of 'Northblue' blueberry. J. Amer. Soc. Hort. Sci. 11:368-371.

Hangarter, R.P., M.D. Peterson, and N.E. Good. 1980. Biological activities of indoleacetylamino acids and their use as auxins in tissue culture. Plant Physiol. 65:761-767.

James, D. 1983. Adventitious root formation in vitro in apple rootstocks *(Malus pumila).* I. Factors affecting the length of the auxin-sensitive phase in M.9 and M.26. Physiol. Plant. 57:154-158.

Leuba, V., D. LeTourneau, and D. Oliver. 1989. Stability of phenpacetic acid in liquid media. J. Plant Growth Regulat. 8:163-165.

Miller, R.G., Jr. 1981. Simultaneous statist&al inference. Springer-Verlag, New York.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Pence, J.D.C. and J.L. Caruso. 1984. Effects of IAA and four IAA conjugates on morphogenesis and callus growth from tomato leaf discs. Plant Cell, Tissue & Organ Cult. 3:101-110.

Srivastava, P.S., A. Steinhauer, and H. Glock. 1985. Plantlet differentiation in leaf and root cultures of birch *(Betula pendula* Roth.). Plant Sci. 42:209-214.

Yamakawa, T., O. Kurahashi, K. Ishida, K. Shigeaki, T. Kodama, and Y. Minoda. 1979. Stability of indole-3-acetic acid to autoclaving, aeration, and light illumination. Agr. Biol. Chem. 43:879-880.