



RESEARCH ARTICLE

# Effect of environmental and cultural conditions in medium pH and plant growth performance of Douglas-fir (*Pseudotsuga menziesii*) shoot culture [version 1; referees: 2 approved with reservations]

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**Abstract**

The medium pH level of plant tissue culture has been shown to be essential to many aspects of explant development and growth. Sensitivity or tolerance of medium pH change *in vitro* varies accordingly to specific requirements of individual species. The objectives of this study are to 1) determine medium pH change over time in storage conditions and with presence of explants, 2) evaluate the effects of medium pH change and explant growth performance and 3) assess the effects of adding a pH stabilizer, 2-(N-morpholino)ethanesulfonic acid (MES) to Douglas-fir micropropagation medium. Spring buds, collected before breaking dormancy from juvenile and mature donor trees were utilized for these evaluations. Medium with or without MES, each at five medium pH levels was pre-adjusted before adding MES, agar and autoclaving. Medium pH changes and explant growth parameters were measured at eight different incubation times. In general, medium with MES provided a more stable medium pH compared to pre-adjusted pH values under two storage conditions as well as with presence of explants over time. A general trend of decreasing medium pH over time was found comparing explants from juvenile and mature donor genotypes. Explant height and weight growth increased over time, but differ among explants from juvenile and mature donor genotypes. MES may help to maintain stable medium pH for bulk medium preparation. Our findings suggested a 21-day subculture practice may facilitate to sustain medium freshness, medium pH level and desirable explant growth.

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report

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## Introduction

The Christmas tree industry plays an important role within Pennsylvania agriculture as well as across the nation. The goal of this micropropagation project was to develop a true-to-type clonal propagation system to alleviate the cost of tree-to-tree variation by conventional seedling propagation. Understanding plant materials and their growing conditions may provide better assistance for later developmental stages in tissue culture.

The medium pH of plant tissue culture has been shown to be very important to many aspects of explant development and growth. Sensitivity or tolerance to medium pH change *in vitro* varies accordingly to specific requirements of individual species. Similar to soil pH, medium pH level may influence nutrient uptake (Ramage & Williams, 2002), cellular pH adjustment (Ballarin-Denti & Antonioti, 1991), rooting and cellular growth (Leifert *et al.*, 1992; de Klerk *et al.*, 2008), plant gene expression and transcriptional pH responses in roots (Lager *et al.*, 2010), and the efficiency of *Agrobacterium*-mediated transformation (Ogaki *et al.*, 2008; Rai *et al.*, 2012). Medium pH also can act to facilitate or inhibit nutrient availability in the medium such as ammonium uptake *in vitro* can be facilitated with a stable pH of 5.5 (Thorpe *et al.*, 2008).

Medium pH fluctuations may be attributed to medium components, autoclaving, ion exchange, and environmental conditions. Medium components may modify pH prior to and after autoclaving (Skirvin *et al.*, 1986; Owen *et al.*, 1991). Organic, inorganic salts, amino acids, vitamins, sucrose, gelling agents, and plant growth regulators are the common components added to tissue culture medium. Williams *et al.* (1990) reported adding agar significantly elevated medium pH prior to autoclaving in group of pre-adjusted pH from 3.5 to 5.5 of MS medium (Murashige & Skoog, 1962) but less increment was found in group of pre-adjusted medium pH from 5.5 to 7.0, or decrease in group of pre-adjusted medium pH from 7.0 to 8.0. In contrast, post-autoclaving medium pH increased in group of pre-adjusted pH of 3.5–4.5 but had more significant medium pH decrease in group of pre-adjusted pH of 5–8. Additions of synthetic or natural organic acids generally increase medium buffering ability (Thorpe *et al.*, 2008). Organic compounds such as 2-(N-morpholino)ethanesulfonic acid (MES) could especially help to maintain suitable medium pH range for explant development (Parfitt *et al.*, 1988; de Klerk *et al.*, 2008; Yuan *et al.*, 2012). MES and vitamin additions were also found to enhance embryo growth during the initiation stage of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) (Franco 1950) somatic embryogenesis (Pullman *et al.*, 2005).

After placing explants on the medium, medium pH fluctuations were also observed for various species. Medium pH of loblolly pine (*Pinus taeda*) grown in liquid suspension medium showed subsequent pH decrease from 5.5 to 4.6 within 5 days of incubation followed by pH increase to 6.0 in 19 days of incubation (Pullman *et al.*, 2005). Significant medium pH decrease was also found after placing mulla mullas (*Ptilotus exaltatus*) shoots on MS medium for 4 weeks (Williams *et al.*, 1990) and an U shaped curve of medium pH fluctuation was found in shoot tip culture of crabapple (*Malus* sp. cv. Almey) and pear (*Pyrus communis* L. cv. Seckel) on MS medium (Singha *et al.*, 1987). According to Skirvin *et al.* (1986) and Thorpe *et al.* (2008), explant nutrient absorption *in vitro* is a function of ion exchange where deposition of free hydrogen ions (H<sup>+</sup>) and hydroxyl ion (OH<sup>-</sup>) in the medium may contribute to acidic

or alkaline medium pH. In contrast, photooxidation induced chelating event bound free iron to reduce iron availability may also influence medium pH (Hangarter & Stasinopoulos, 1991). Secretion of plant secondary metabolites into culture medium is common *in vitro* (Dörnenburg & Knorr, 1995) but its role and function in altering medium pH and nutrient absorption are not clear.

Medium pH fluctuations can involve many factors, and could eventually become problematic if lacking of care attentions. Douglas-fir shoot culture can be grown in a modified version (mDCR) of Douglas-fir cotyledon revised medium (DCR, Gupta & Durzan, 1985; Gupta & Durzan, 1987a). However, the interactions between the species and its growing medium are not well understood. To ensure an optimal shoot culture development and provide high quality shoots for later development of rooting protocol, medium pH can be a key indicator in determine subculture time. It may also be further utilized as a diagnostic tool for some abnormal growth symptoms such as necrosis, caused by low pH induced nutrient deficiency. The experimental system may be applied to studies of explant development and nutrient relationship *in vitro* (Singha *et al.*, 1990). Hence, the objectives of this study are to 1) determine medium pH change over various times under storage conditions and in the presence of explants, 2) evaluate the effects of prolonged culturing on medium pH and explant growth performance, and 3) assess the effects of addition of a pH stabilizer, MES to Douglas-fir micropropagation medium.

## Materials and methods

Spring buds from juvenile (HF205 and HF210) and mature (PS-2) donor trees collected in 2006 were utilized for this study. These bud samples were collected prior to breaking dormancy. To classify juvenility, Douglas-fir trees of Lincoln seed source planted 10 years ago and not yet producing cones at the Penn State Horticulture Research Farm of Russell E. Larson Agricultural Research and Education Center at Rock Springs were the selected donors. The selected mature donor tree, as a result from a previous genetics study conducted by Gerhold (1984) was over 40 years old at the Penn State golf course. These bud samples were stored in a 4°C cold room until further culture initiation. Preparation, sterilization, and dissection of collected bud sample were followed as per Traore *et al.* (2005).

This study consisted of a four-factor factorial design with three replications for each factor combination. Two juvenile genotypes, HF205 and HF210, and one mature genotype, PS-2 were entered for evaluations. Two types of media were used including mDCR only and mDCR with 2 g/L of MES (mDCR+MES) (M3671, Sigma-Aldrich, St. Louis, MO, USA). Levels of media pH were pre-adjusted to 3.6, 5.1, 5.7, 6.3, and 7.8 before adding 7 g of agar, MES, and autoclaving. Five dissected vegetative buds from each genotype were placed on each treatment combination. Controls consisted of medium without the presence of explants, at each pH level. They were placed in full dark versus light conditions in 25°C growth chambers. For treatments, explants were dissected and placed into mDCR versus mDCR+MES media for incubation for 1, 3, 5, 7, 14, 21, 28, and 35 days.

During the dissection process, measurements of samples were taken for initial bud weight (mg) and petri dish (PD) weight (mg) with solidified media. After each treatment and incubation time, final

media pH, final PD weight (mg), and explant final weight (mg) were recorded. Explant weight change (mg) was obtained by subtracting the initial bud weights from final explant weights. Medium pH was measured at five positions in the plates, between the explants, using a Thermo Orion PerpHecT pH meter (Thermo Fisher Scientific Inc., Waltham, MA, USA). Data analyses were performed using Minitab (Minitab Inc., State College, PA, USA), and graphs were generated by SigmaPlot (Systat Software Inc., Chicago, IL, USA), including ANOVA General Linear Model (GLM), Tukey Honestly Significant Difference test (HSD), and linear regression with significance set at  $p < 0.05$ .

## Results

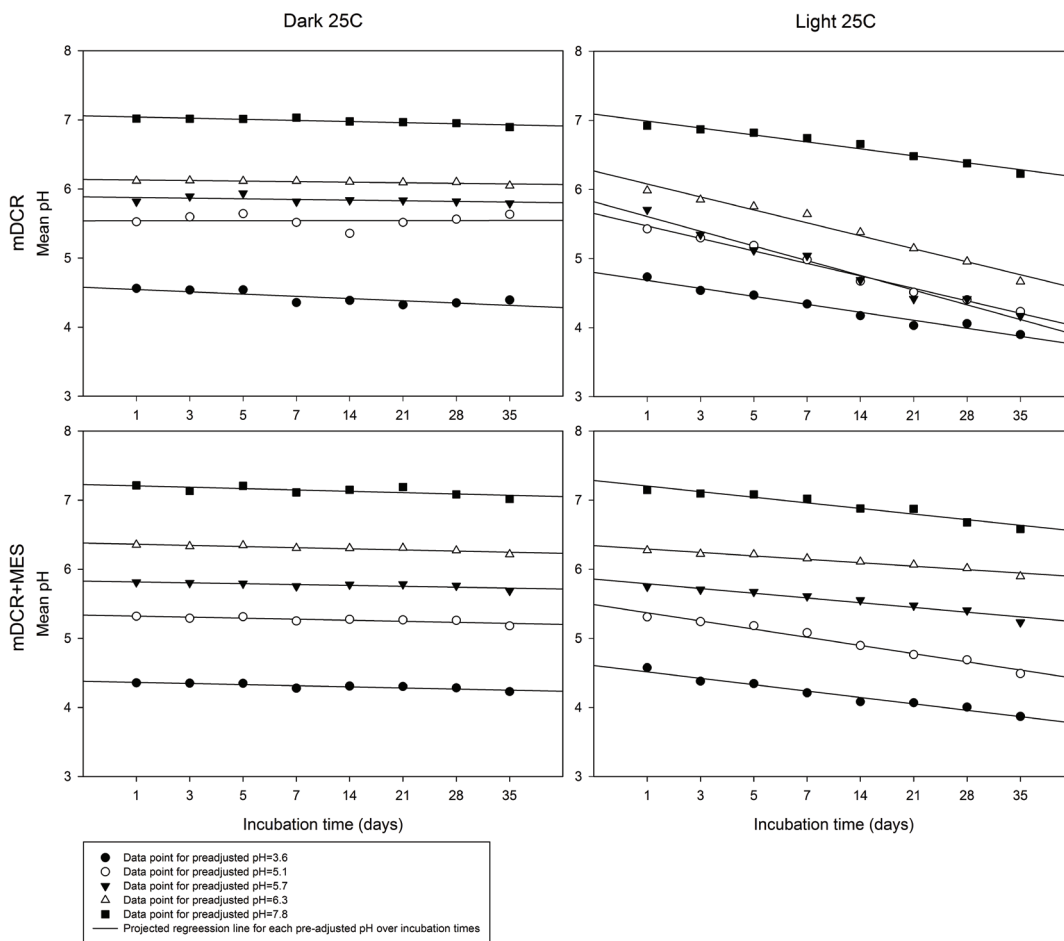
### Media pH and explant weight changes over incubation times for Douglas-fir (*Pseudotsuga menziesii*) shoot cultures grown on different media

4 Data Files

<http://dx.doi.org/10.6084/m9.figshare.1257689>

### Culture medium pH change after autoclaving and storage

After autoclaving, media pH shifts were found according to each pre-adjusted media pH. From 100 samples, mDCR medium showed a greater extent of pH fluctuations than mDCR+MES post-autoclaving. Media initially set at pH 3.6, 5.1, and 5.7 were increased for both media type (pH changes of 0.83, 0.58, 0.17 and 0.76, 0.22, 0.11 for mDCR and mDCR+MES, respectively) whereas medium initially at pH 7.8 were decreased (-0.66 and -0.59 for mDCR and mDCR+MES, respectively). Medium of pre-adjusted pH 6.3 showed decrease in mDCR (-0.11) but increase for mDCR+MES (0.05). In general, dark storage was better to maintain stable media pH than storage in light. Overall, media pH was 5.8 ( $n=400$ ) when kept in the dark condition compared to pH 5.4 ( $n=400$ ) in the light ( $P=0.000$ ). Over the incubation times tested, media pH was maintained at fairly stable condition with a slight decreasing trend in the dark storage condition. In contrast, media pH had a stronger decreasing trend when incubated in the light. The mDCR+MES media maintained media with less pH change over the incubation times when compared to mDCR media in the light (Figure 1).

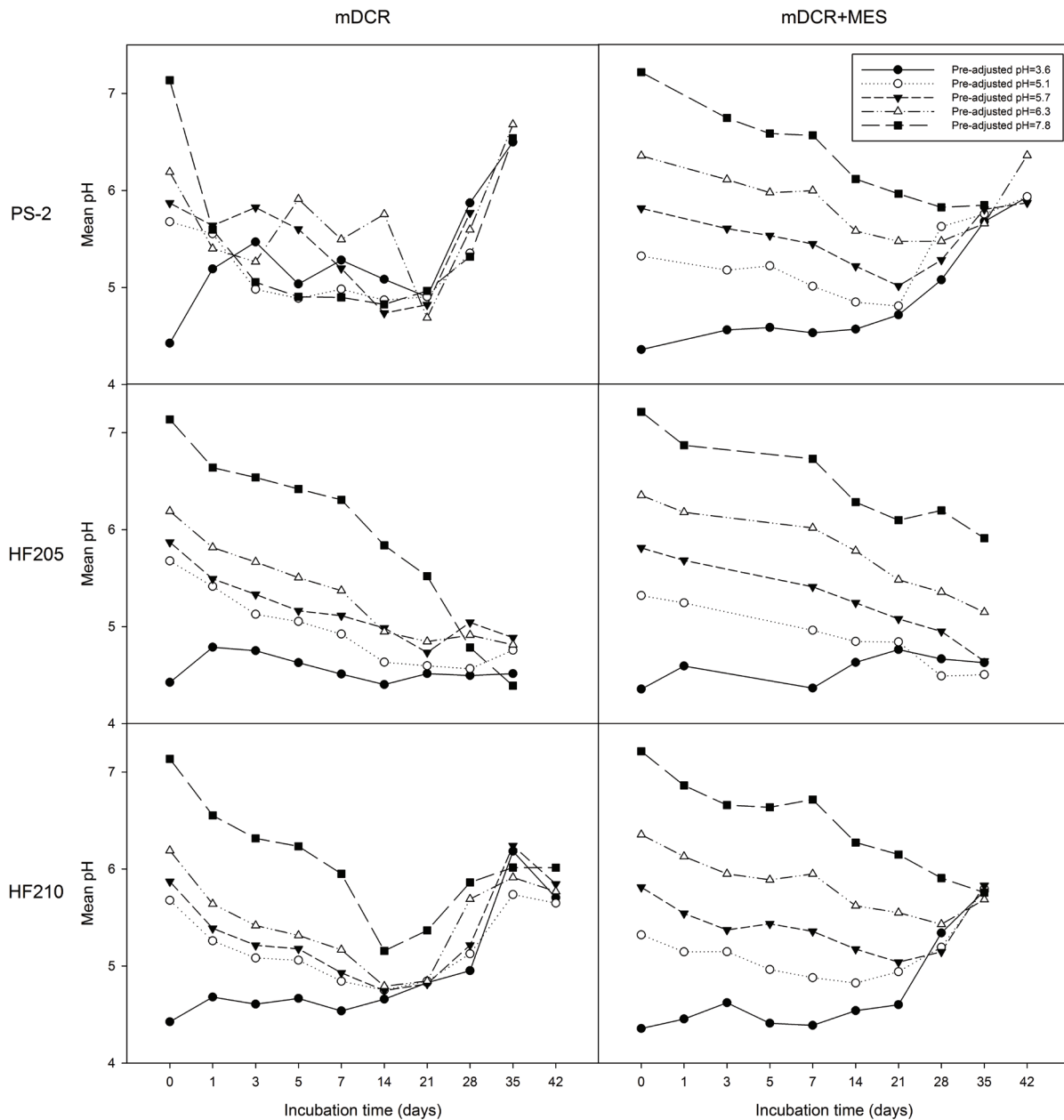


**Figure 1. Media pH changes over incubation times from mDCR and mDCR+MES media incubated under dark and light conditions.** Medium pH was pre-adjusted to 3.6, 5.1, 5.7, 6.3, and 7.8 prior to adding 7 g/L of agar, MES and autoclave. Medium was incubated in a growth chamber with full light or darkness at 25°C until it reached incubation requirement. Post-autoclaving pH was recorded using a pH meter at each incubation time. Datapoints represent mean pH ( $n=5$  for each datapoint), and were fitted with linear regression lines. Please see Dataset 1 for the raw data.

### The effect of MES on medium pH

After placing explants into the media, the pH of the medium was significantly influenced by all factors, genotype, media type, initial pH level, and incubation time (all  $P=0.000$ ). Overall medium pH was 5.45 from medium incubated with PS-2 ( $n=1,355$ ), which significantly greater than 5.41 and 5.19 from those incubated with HF210 ( $n=1,384$ ) and HF205 ( $n=950$ ), respectively. The medium pH from mDCR+MES ( $n=1,805$ ) was significantly greater than the pH of mDCR only medium ( $n=1,884$ ) (5.45 vs. 5.28, respectively)

( $P=0.000$ ). Decreasing media pH over incubation time and variation among genotypes were both observed. Media with addition of MES was better able to maintain stable media pH up to 21 days of incubation from each of the 5 different initial pH levels (Figure 2). Regardless of initial pH level, incubation time had a strong significant effect on media pH ( $P=0.000$ ). Combined two types of media, mean medium pH showed the lowest value of 5.04 ( $n=435$ ) at 21-day of incubation. In contrast, the highest mean medium pH 5.88 was recorded at the 42-day of incubation ( $n=125$ ). Overall

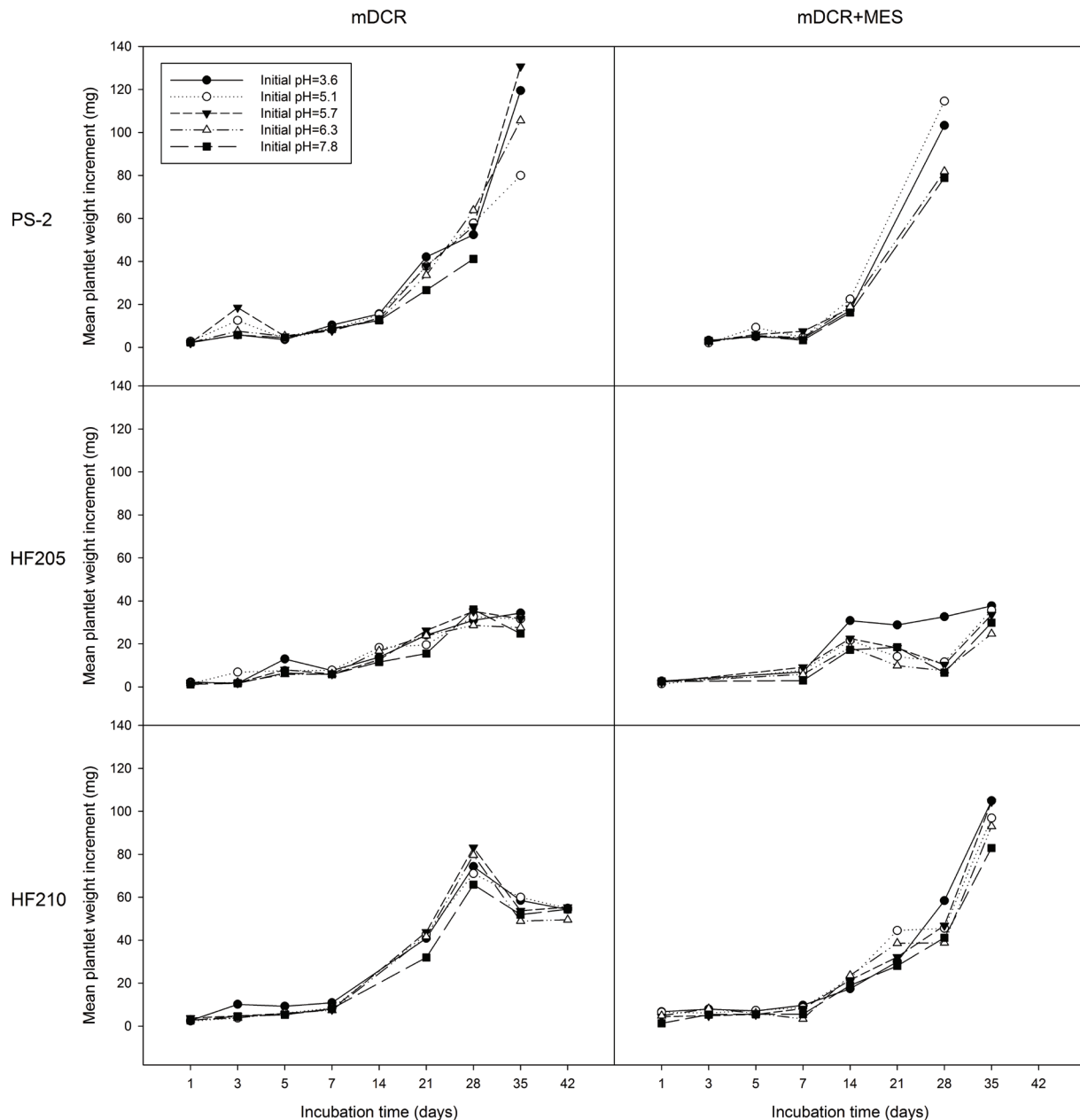


**Figure 2. Medium pH changes over incubation times from three genotypes in mDCR and mDCR+MES media.** Genotypes included one mature genotype, PS-2 and two juvenile genotypes, HF205 and HF210. After surface sterilization and dissection, inner vegetative buds from above three genotypes were placed on either mDCR or mDCR+MES medium for incubation up to 42 days. These buds were incubated in a growth chamber at 25°C with light regime adjusted to 16-hour light followed by 8-hour darkness each day. Medium pH was recorded using a pH meter when sample reached each incubation requirement where five pH values were recorded within each petri dish. Datapoints represent mean pH. Please see [Dataset 2](#) and [3](#) for the raw data.

medium pH at each initial pH (3.6, 5.1, 5.7, 6.3, and 7.8) showed significant differences between each other including 4.90 (n=744), 5.08 (n=740), 5.30 (n=745), 5.57 (n=740), and 5.99 (n=720), respectively. The media pH stabilizer MES demonstrated its ability to prevent media pH from dropping at the higher or lower ends of initial pH levels. Within individual genotype, both media type, and incubation time showed significant effects on media pH for all genotypes ( $P=0.000$ ).

### The effect of MES on plant growth

For explants growth response, genotype ( $P=0.000$ ), incubation time ( $P=0.000$ ), and initial pH ( $P=0.012$ ) showed significant effects on explant weight increment (mg). The addition of MES into the media did not show a significant effect on explant weight increment ( $P=0.281$ ) (Figure 3). Overall, HF210 (30.86 mg, n=264) and PS-2 (22.50 mg, n=190) had a significant weight increment greater than HF205 (13.83 mg, n=205) ( $P=0.000$ ). Explant weight increment



**Figure 3.** Mean explant weight increment changes (mg) among genotypes and medium types at each initial pH level. Genotypes included one mature genotype, PS-2 and two juvenile genotypes, HF205 and HF210. After surface sterilization and dissection, inner vegetative buds from above three genotypes were placed on either mDCR or mDCR+MES medium at each initial pH level (3.6, 5.1, 5.7, 6.3, and 7.8) for incubation up to 42 days. These buds were incubated in a growth chamber at 25°C with light regime adjusted to 16-hour light followed by 8-hour darkness each day. Bud weight change was recorded using an electronic scale when sample reached each incubation requirement. Datapoints represent mean weight change (mg). Please see [Dataset 4](#) for the raw data.



of HF210 did not show a significant difference when compared with PS-2 ( $P=0.2903$ ). However, a distinct trend in explant weight decrease was observed after 28 days of incubation in mDCR only medium for HF210. Initial medium pH of 3.6 had a significantly greater bud weight change ( $P=0.005$ ) than medium pH 7.8 (25.89 vs. 19.23 mg,  $n=134$  vs. 130, respectively). An increasing trend of bud weight change was observed but bud weight did not show significant increase during the first week of incubation.

Comparing individual genotypes, medium type exhibited non-significant effect on explant weight increment of all three genotypes ( $P>0.05$ ). For HF205, the weight differences were observed at the higher and lower ends of the given initial pH levels (Figure 4). Incubation time showed significant effect on explant weight increment for all three genotypes ( $P=0.000$ ). For all three genotypes, explant weight did not show any significant differences for the first 7 days of incubation. However afterwards, explant weight growth dramatically increased for PS-2 and HF210. HF205 showed much less weight increment than the other two genotypes (Figure 5).

#### Morphological observations of explants

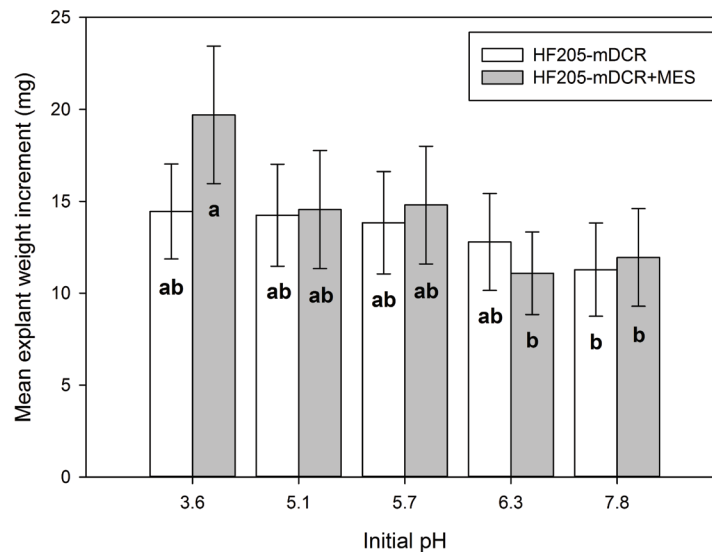
After growing in medium for 28-days or more without being sub-cultured, explants showed various growth deformities such as chlorosis, delayed needle expansion, tip browning, browning of the bottom of explants and surrounding medium, vitrification, and even death. These symptoms occurred especially at the lower and higher ends of the initial pH levels, after prolonged culturing. Regardless of the given initial pH levels, explant growth did not show obvious delay at the early culture stages. The mentioned problems were only

found at the later times of culturing. Moisture condensation was a common problem in the plate-based tissue culture system. Some of the deformities observed may have been associated with excessive amount of water droplets falling onto the medium surface or coming into contact with the explants.

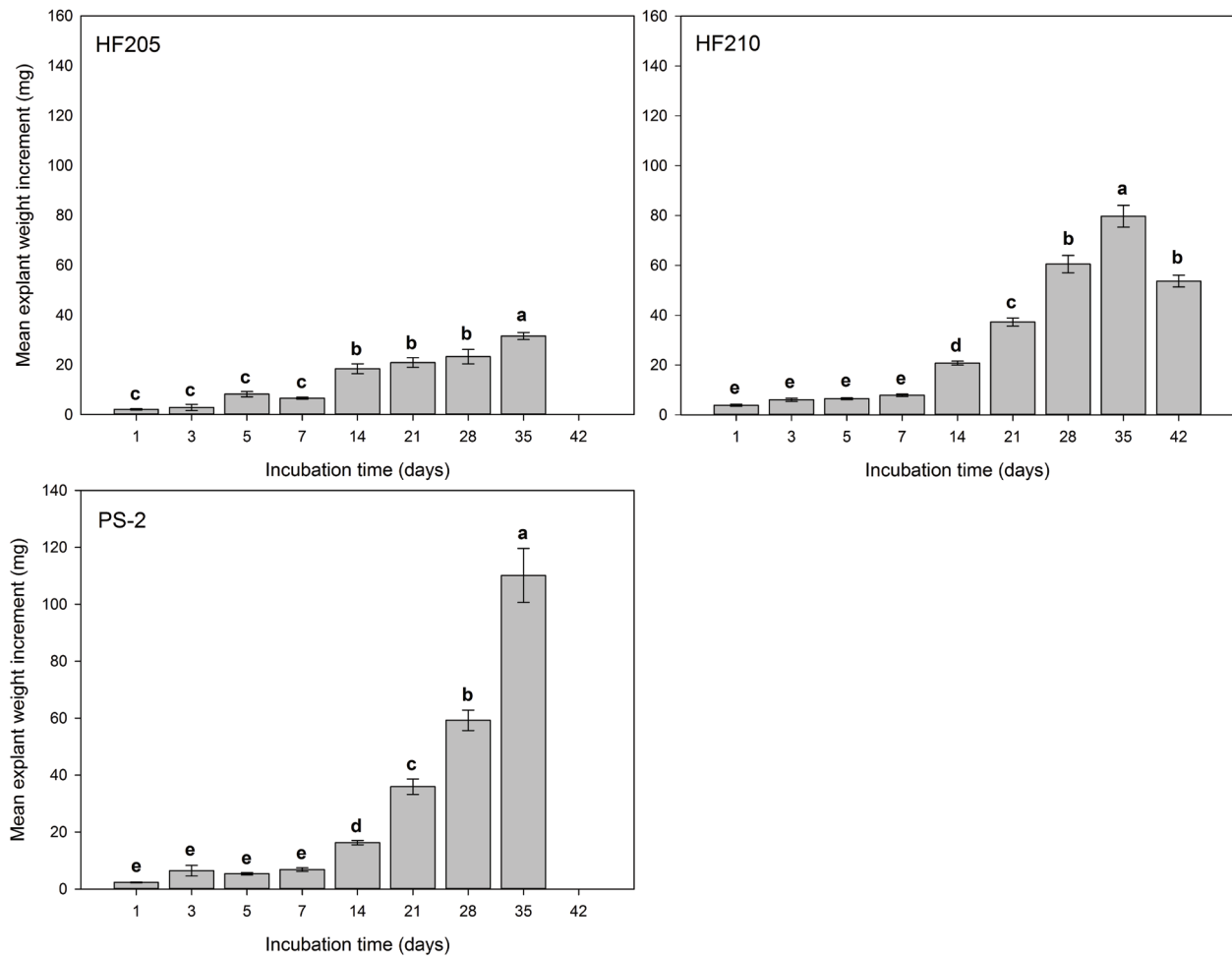
#### Discussion

In general, mDCR medium with MES provided more stability of the pre-adjusted pH values after autoclaving in both the absence and presence of explants in the medium. We observed that after autoclaving, medium pH changed, but mDCR medium with MES showed less medium pH fluctuation than without MES. Storage of medium in the dark resulted in less medium pH fluctuation than storage under light. For mDCR medium incubated with explants, pH showed a gradual decrease that was followed by a sharp increase over the incubation time. However, mDCR+MES medium exhibited a slower decrease in pH or was followed by a convergent medium pH change for all pre-adjusted pH levels. Explant weight gain over time showed an inverse relationship with medium pH change, but also differed between juvenile and mature genotypes. The addition of MES did not show significant influence on explant weight growth. However, a distinct decrease in explant weight growth was observed after 28 days of incubation in the mDCR only medium.

Medium storage is a common practice in tissue culture. The use of premade medium serves two main purposes. One is to hold the medium for a period of time to observe whether any contamination occurs in the medium. This ensures maximum explant growth performance achieved when antibiotics are not present in the medium.



**Figure 4. Mean explant weight increment changes (mg) among medium types at each initial pH level from HF205.** After surface sterilization and dissection, inner vegetative buds from HF205 were placed on either mDCR or mDCR+MES medium at each initial pH level (3.6, 5.1, 5.7, 6.3, and 7.8) for incubation up to 42 days. These buds were incubated in a growth chamber at 25°C with light regime adjusted to 16-hour light followed by 8-hour darkness each day. Bud weight change was recorded using an electronic scale when sample reached each incubation requirement. Vertical bars represent mean weight change (mg)±S.E. Means sharing the same letter indicate non-significant difference between means ( $P>0.05$ ). Tukey's (HSD) multiple comparison was used.



**Figure 5. Mean explant weight changes (mg) over incubation times from PS-2, HF205 and HF210.** After surface sterilization and dissection, inner vegetative buds from three genotypes were placed on either mDCR or mDCR+MES medium at each initial pH level (3.6, 5.1, 5.7, 6.3, and 7.8) for incubation up to 42 days. These buds were incubated in a growth chamber at 25°C with light regime adjusted to 16-hour light followed by 8-hour darkness each day. Bud weight change was recorded using an electronic scale when sample reached each incubation requirement. Vertical bars represent mean weight change (mg)±S.E. Sample sizes according to the incubation time (1–42 days) were for HF205, n=45, 15, 15, 30, 30, 20, 20, and 30, for HF210, n=30, 30, 30, 30, 30, 31, 30, 38, and 15, and for PS-2, n=15, 30, 30, 30, 30, 16, 33, and 6. Means sharing the same letter indicate non-significant difference between means ( $P>0.05$ ). Tukey's (HSD) multiple comparison was used. Please see [Dataset 4](#) for the raw data.

The other purpose is to facilitate timely arrangements of routine culture initiations and transfers. *Owen et al. (1991)* demonstrated that light affects post-autoclave medium storage, resulting in reduced medium pH over storage time. Our data confirmed their findings. Medium pH was more stable in the dark storage condition regardless of the presence of MES. The addition of MES could stabilize medium pH under the light condition, in both the presence and absence of explants.

Fluctuation of medium pH can be influenced by many factors. Hydrolysis, enzymatic break down, photooxidation and photolysis on light-sensitive medium components may all contribute to the fluctuation of medium pH. Sucrose hydrolysis often requires splitting of water molecules and breaking glycosidic bonds of the disaccharides. Once the breakage of glycosidic bonds has occurred, hydrogen

ions from the splitting of water molecules bind with glucose, whereas hydroxyl groups bind with fructose. Since tissue culture medium often is adjusted to slight acidic conditions (pH 5.2–5.8), autoclaving provides a suitable temperature for catalyzing sucrose hydrolysis. Acid facilitated autocatalyzed sucrose hydrolysis was reported as being both pH and temperature dependant, where lower pH at a given temperature promotes more sucrose hydrolysis (*Heidt et al., 1952; Wann et al., 1997*). The availability of hydrogen ions in the acidic medium solution also depends on the buffering ability of the nutrient components (*Thorpe et al., 2008*). Furthermore, carbon sources, the amount of carbohydrates, and gelling agents act together to determine the amount of sucrose hydrolysis and the medium pH after autoclaving. As a result, medium with lower original pH may become higher while medium with higher original pH may become lower to reach equilibrium of the solution.



After explants are introduced into the medium vessels, the sucrose is further converted into monosaccharides inter- and intra-cellularly by invertase or other plant enzymes (Thorpe *et al.*, 2008). Egger & Hampp (1993) found the optimum activity of soluble acid invertase was at pH 4.1 in developing spruce (*Picea abies* (L.) Karst.) needles. They also reported that other sucrose synthesis enzymes, sucrose phosphate synthase, and sucrose synthase were pH dependant for their optimal activities (pH 7.7 and 6.7, respectively). Hence, as explants host numerous biological activities they must balance pH levels accordingly for each of the biochemical reactions. Ion uptake and release become the mechanism by which cells adjust for pH requirements. Dodds & Roberts (1995) attributed the fluctuation of medium pH after autoclaving may be a result of imbalance of anion and cation uptake.

Photochemistry may further induce degradation of photo-sensitive compounds in the medium, and trigger pH fluctuation. Photolysis is an event introduced by photons. For example, when a water molecule receives energy from photons during the photosynthesis process, photolysis occurs to generate electrons, hydrogen ions, and oxygen. If the free hydrogen ions are not bound by other substrates and excreted into the medium, they may cause medium pH fluctuation. Hangarter & Stasinopoulos (1991) reported a light induced Fe-catalyzed photooxidation of EDTA, which caused reduced root growth of the *Arabidopsis thaliana* ecotype Columbia. EDTA, an ion chelator, is considered to be a buffering agent in the tissue culture medium. After photooxidation occurred, formaldehyde and glyoxylic acid are produced, which can be toxic to explants, concomitant with increased chelated ferric oxide that explants can not readily use. This light induced change of buffering ability could definitely alter nutrient availability in the medium, and further affect the fluctuation of medium pH.

MES can be utilized to stabilize medium pH for Douglas-fir micro-propagation. MES has been employed in various tissue culture systems to maintain stable medium pH over extended cultural times. Park & Son (1992) reported the addition of MES alone in the medium, or together with Dithiothreitol, increased protoplast yield and viability from hybrid poplar protoplast culture system. Similarly, MES and arabinogalactan-protein, alone or combined, were found to maintain suitable medium pH and to enhance embryogenesis in white cabbage (*Brassica oleracea* var. *capitata*) microspore culture system (Yuan *et al.*, 2012). For conifer species, MES was also utilized as pH stabilizer for silver fir (*Abies alba* L.) (Hartmann *et al.*, 1992) and European larch (*Larix decidua* Mill.) (Korlach & Zoglauer, 1995) protoplast culture systems. Protoplasts, being a single cell without cell wall, are probably extremely sensitive to

rapid pH changes. The effects of pH changes could be as important for shoot culture or other tissue culture prospects for explant productivity. Especially, MES may help to maintain stable medium pH for bulk medium preparation in large scale aspects of a propagation project.

Our data suggested a 21-day subculture practice may be suitable for maintaining medium freshness, medium pH level, and desirable explant growth for Douglas-fir shoot culture. Although there might be specific pH requirements for individual species, explants of Douglas-fir genotypes showed various responses or adaptations to medium pH changes. Some genotypes may be able to tolerate or adapt better to fluctuations in medium pH, and to show continuous growth in a wide range of pH levels. The effects of MES and nutrient acquisition by explants in culture may require further investigations on specific aspects of nutrient dynamics regarding the effects of both medium and explants *in vitro*.

### Data availability

Figshare: <http://dx.doi.org/10.6084/m9.figshare.1257689> (Chen *et al.*, 2014).

### Author contributions

CCC performed the experiments, analyzed the data, and wrote the first draft of this manuscript. All authors contributed equally in data interpretations as well as writing and further refinement of the manuscript.

### Competing interests

No competing interests were disclosed.

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## References

- Ballarin-Denti A, Antoniotti D: **An experimental approach to pH measurement in the intercellular free space of higher plant tissues.** *Experientia*. 1991; 47(5): 478–482.  
[Publisher Full Text](#)
- Chen CC, Bates R, Carlson J: **Media pH and explant weight changes over incubation times for Douglas-fir (*Pseudotsuga menziesii*) shoot cultures grown on different media.** *Figshare*. 2014.  
[Data Source](#)

- Dodds JH, Roberts LW: **Experiments in plant tissue culture.** (Cambridge University Press, New York). 1995.  
[Reference Source](#)
- Dörnenburg H, Knorr D: **Strategies for the improvement of secondary metabolite production in plant cell cultures.** *Enzym Microb Technol*. 1995; 17(8): 674–684.  
[Publisher Full Text](#)
- de Klerk G-J, Hanecakova J, Jaski J: **Effect of medium-pH and MES on**

- adventitious root formation from stem disks of apple. *Plant Cell Tiss Organ Cult*. 2008; **95**(3): 285–292.  
[Publisher Full Text](#)
- Egger B, Hampp R: Invertase, sucrose synthase and sucrose phosphate synthase in lyophilized spruce needles; microplate reader assays. *Trees*. 1993; **7**(6): 98–103.  
[Publisher Full Text](#)
- Gerhold HD: Transferring genetic research results to users: Pennsylvania State collaborates with Pennsylvania-TIP [Tree Improvement Program, *Pinus sylvestris*, *Pseudotsuga menziesii*]. *Am Christmas Tree J (USA)*. 1984; **28**(2): 51–54.  
[Reference Source](#)
- Gupta PK, Durzan DJ: Shoot multiplication from mature trees of Douglas-fir (*Pseudotsuga menziesii*) and sugar pine (*Pinus lambertiana*). *Plant Cell Rep*. 1985; **4**(4): 177–179.  
[PubMed Abstract](#) | [Publisher Full Text](#)
- Gupta PK, Durzan DJ: *In vitro* establishment and multiplication of juvenile and mature Douglas-fir and sugar pine. *Acta Hortic*. 1987a; **212**: 483–487.  
[Reference Source](#)
- Gupta PK, Durzan DJ: Micropropagation and phase specificity in mature elite Douglas-fir. *J Am Soc Hortic Sci*. 1987b; **112**(6): 969–971.  
[Reference Source](#)
- Hangarter RP, Stasinopoulos TC: Effect of Fe-catalyzed photooxidation of EDTA on root growth in plant culture media. *Plant Physiol*. 1991; **96**(3): 843–847.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Hartmann S, Lang H, Reuther G: Differentiation of somatic embryos from protoplasts isolated from embryogenic suspension cultures of *Abies alba* L. *Plant Cell Rep*. 1992; **11**(11): 554–557.  
[PubMed Abstract](#) | [Publisher Full Text](#)
- Heidt LJ, William Southam F, Sullivan EA: Autocatalyzed hydrolysis of sucrose by acid. *J Am Chem Soc*. 1952; **74**(9): 2377–2378  
[Publisher Full Text](#)
- Korlach J, Zoglauer K: Developmental patterns during direct somatic embryogenesis in protoplast cultures of European larch (*Larix decidua* Mill.). *Plant Cell Rep*. 1995; **15**(3–4): 242–247.  
[PubMed Abstract](#) | [Publisher Full Text](#)
- Leifert C, Pryce S, Lumsden PJ, et al.: Effect of medium acidity on growth and rooting of different plant species growing *in vitro*. *Plant Cell Tiss Organ Cult*. 1992; **30**(3): 171–179.  
[Publisher Full Text](#)
- Lager I, Andreasson O, Dunbar TL, et al.: Changes in external pH rapidly alter plant gene expression and modulate auxin and elicitor responses. *Plant Cell Environ*. 2010; **33**(9): 1513–1528.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Murashige T, Skoog F: A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant*. 1962; **15**(3): 473–497.  
[Publisher Full Text](#)
- Owen HR, Wengerd D, Miller AR: Culture medium pH is influenced by basal medium, carbohydrate source, gelling agent, activated charcoal, and medium storage method. *Plant Cell Rep*. 1991; **10**(11): 583–586.  
[PubMed Abstract](#) | [Publisher Full Text](#)
- Okaki M, Furuichi Y, Kuroda K, et al.: Importance of co-cultivation medium pH for successful *Agrobacterium*-mediated transformation of *Lilium x formolongi*. *Plant Cell Rep*. 2008; **27**(4): 699–705.  
[PubMed Abstract](#) | [Publisher Full Text](#)
- Parfitt DE, Almehdi AA, Bloksberg LN: Use of organic buffers in plant tissue-culture systems. *Sci Hortic*. 1988; **36**(3–4): 157–163.  
[Publisher Full Text](#)
- Park YG, Son SH: *In vitro* shoot regeneration from leaf mesophyll protoplasts of hybrid poplar (*Populus nigra* x *P. maximowiczii*). *Plant Cell Rep*. 1992; **11**(1): 2–6.  
[PubMed Abstract](#) | [Publisher Full Text](#)
- Pullman GS, Johnson S, Van Tassel S, et al.: Somatic embryogenesis in loblolly pine (*Pinus taeda*) and Douglas fir (*Pseudotsuga menziesii*): improving culture initiation and growth with MES pH buffer, biotin, and folic acid. *Plant Cell Tiss Organ Cult*. 2005; **80**(1): 91–103.  
[Publisher Full Text](#)
- Ramage CM, Williams RR: Mineral nutrition and plant morphogenesis. *In Vitro Cell Dev Biol - Plant*. 2002; **38**(2): 116–124.  
[Publisher Full Text](#)
- Rai GK, Rai NP, Kumar S, et al.: Effects of explant age germination medium, pre-culture parameters, inoculation medium, pH washing medium, and selection regime on *Agrobacterium*-mediated transformation of tomato. *In Vitro Cell Dev Biol - Plant*. 2012; **48**(5): 565–578.  
[Publisher Full Text](#)
- Singha S, Oberly GH, Townsend EC: Changes in nutrient composition and pH of the culture medium during *in vitro* shoot proliferation of crabapple and pear. *Plant Cell Tiss Organ Cult*. 1987; **11**(3): 209–220.  
[Publisher Full Text](#)
- Skirvin RM, Chu MC, Mann ML, et al.: Stability of tissue culture medium pH as a function of autoclaving, time, and cultured plant material. *Plant Cell Rep*. 1986; **5**(4): 292–294.  
[PubMed Abstract](#) | [Publisher Full Text](#)
- Thorpe T, Stasolla C, Yeung EC, et al.: The components of plant tissue culture media II: organic additions, osmotic and pH effects, and support systems. In: George EF et al. (ed), *Plant propagation by tissue culture*, 3rd edn volume 1, the background. (Springer, Dordrecht, The Netherlands). 2008; 115–173.  
[Publisher Full Text](#)
- Traore A, Xing Z, Bonser AL, et al.: Optimizing a protocol for sterilization and *in vitro* establishment of vegetative buds from mature Douglas-fir tree. *Hortsci*. 2005; **40**(5): 1464–1468.  
[Reference Source](#)
- Wann SR, Veazey RL, Kaphammer J: Activated charcoal does not catalyze sucrose hydrolysis in tissue culture media during autoclaving. *Plant Cell Tiss Organ Cult*. 1997; **50**(3): 221–224.  
[Publisher Full Text](#)
- Williams RR, Taji AM, Winney KA: The effect of Ptilotus plant tissue on pH of *in vitro* media. *Plant Cell Tiss Organ Cult*. 1990; **22**(22): 153–158.  
[Publisher Full Text](#)
- Yuan S-X, Su Y-B, Liu Y-M, et al.: Effects of pH, MES, arabinogalactan-proteins on microspore cultures in white cabbage. *Plant Cell Tiss Organ Cult*. 2012; **110**(1): 69–76.  
[Publisher Full Text](#)

# Open Peer Review

Current Referee Status:



Version 1

Referee Report 10 February 2015

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**Isabel Arrillaga**

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The effect of the pH in the culture medium is a very important factor that often is not taken into account. This papers reports on the changes in the medium pH at several times during the tissue culture process and their effect on the explants growth. From this point of view the article is very interesting and deserves to be accepted for publication. Changes in the pH of the culture medium after and before autoclaving and during culture time have been studied in depth (see any of the editions of the book series Plant Propagation by Tissue Culture, George and Sherrington, Exegetics, London).

The paper is well written and data well analyzed. I have found very interesting results as the effect of light on the medium pH during storage that can be of interest for plant tissue colleagues.

Also, I have some questions to address to the authors.

1. Why to use pH below 5?. Most of the culture media are adjusted to a pH from 5,7- to 6,0 before autoclaving.
2. Did authors find any problems with low pH and agar gelification?. In our experience low pH affect media solidification.
3. The effect of MES on medium pH stability is expected.
4. From your data on Figures 3 and 5, I should not agree with your statement “21-day subculture practice may facilitate to sustain medium freshness, medium pH and desirable explant growth” (Abstract). It is true that pH of the medium increased after 21 of explant culture initiation, but it is also true that irrespective of the medium pH, explants sharply increased weight after 21 days in culture. These results should be emphasized and conclusions rewritten.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

**Competing Interests:** No competing interests were disclosed.

Author Response 10 Feb 2015

**Chien-Chih Chen**, Clemson University, USA

Dr. Arrillaga, thank you so much for your comments. We are now working on the revision of this manuscript addressing reviewers' concerns.

**Competing Interests:** No competing interests were disclosed.

Author Response 29 Apr 2015

**Chien-Chih Chen**, Clemson University, USA

We appreciate your comments. Please find our explanations regarding your concerns with the original version (and how they have been addressed in the new version of the article) below.

1. The reason that we chose the larger range of pH levels was to expand upon observations from our routine practice in tissue culture medium preparation. Especially, this was to understand how Douglas-fir explants would respond to various levels of medium pH *in vitro*, while the 5.7 to 6.0 range derives more from *in vivo* conditions. The medium pH and explant weight increment curves provide detailed records relevant to both storage and tissue culture conditions, which we feel also expands upon the range of observations normally reported.
2. You are correct. Low medium pH can affect agar gelling, and this is associated with types of gelling agents used and other additives (i.e. high concentrations of PEG) as well. We did have an issue with gelling, but not until the pre-adjusted medium pH was below pH 3.6 in our case.
3. We agree that MES is a commonly used buffer in tissue culture. Because the pKa for MES is 6.15 at 25 °C, we felt it was necessary to observe what the buffering capacity of MES was with starting pH levels far from 6.15 that we wanted to investigate in medium under storage and with explants. If MES is added, what medium pH can be maintained, and for what duration, at such extreme pH levels under our test conditions?
4. This is a very good point. As you can see from Fig. 3 and 5, explant weight increment according to various levels of pre-adjusted medium pH was genotype dependent. A broad evaluation including more genotypes would provide a more strong indication. Usually, explant weight increment was in a curvilinear relationship. We did observe a decreasing weight gain in HF210 after 28 days. Also based on Fig.2 and morphological observations, prolonged culture without subculturing could result in additional growth complications. Therefore, we would like to suggest a 21-day subculturing practice as optimal for Douglas-fir explants taking all the various factors and observations into account. We will provide a more precise conclusion in our revision.

**Competing Interests:** No competing interests were disclosed.

Referee Report 06 February 2015

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**Vibha Srivastava**

Department of Crop, Soil, and Environmental Sciences, University of Arkansas, Fayetteville, USA

This is a descriptive paper analyzing pH fluctuations in the tissue culture media for the micropropagation of Douglas fir, and assessing the effect of MES, a commonly used compound in tissue culture media, for stabilizing media pH. Parameters such as the effect of incubation time (days) and dark/light in the presence or absence of MES were studied, in addition to the morphology of explant (buds) in MES + or - media. As expected, MES was found to be useful in stabilizing pH in different conditions and therefore recommended for the Douglas fir micropropagation media. This paper could be useful to the horticulturists, especially those working on micropropagation; though, the findings of this paper, in my opinion, are common knowledge in the field of tissue culture.

I have one major criticism on the experimental design and data analysis, which compels me to approve this paper with reservations: only one concentration, 2 g/L, of MES was used throughout this study. No reasoning was presented towards selection of this particular concentration. MES is used in many tissue culture media at different concentrations. While the authors shy away from making any conclusions (regarding the role of MES), they suggest MES played a role in stabilizing pH. This conclusion cannot be substantiated without a dose-response curve and determination of optimum concentration.

A minor criticism that I have is related to description in materials and methods. The tissue culture media used through out this study is simply referred by the acronym, mDCR. But no description of this media given is given, only reference provided. Whether it is MS/B5/N6 based media is not clear from this paper, and which other compounds are present in this media is not described.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

**Competing Interests:** No competing interests were disclosed.

Author Response 06 Feb 2015

**Chien-Chih Chen**, Clemson University, USA

We would like to thank you for your comments on enhancing this manuscript. Once we received more reviews, we will include your suggestions and revise this manuscript.

**Competing Interests:** No competing interests were disclosed.No competing interests were disclosed.

Author Response 29 Apr 2015

**Chien-Chih Chen**, Clemson University, USA

We appreciate your comments. Please find our explanations regarding your concerns with the original version (and how they have been addressed in the new version of the article) below.

1. We agree that MES is commonly used in tissue culture. It would have been better to establish a dose-response curve and determine the optimal concentration for our experimental system. But that would have involved testing the MES range with all of the starting pH levels and storage conditions and multiple explant genotypes. However, we were limited in the number of explant samples available from the Christmas tree seed orchard, and over the course of the research we did not have enough material to investigate

all of the possible multi-factorial combinations. Instead, since we were mostly interested in learning how medium pH changes under storage and culture conditions, and how that effects the explant growth responses, we started with one MES level previously reported with conifer explant culture. As expected, that level of MES did maintain a better medium pH. This study was at the beginning of a long series of experiments to establish a protocol for an effective micropropagation method for a recalcitrant woody species, Douglas-fir, for large scale application. Because the one concentration of MES worked well, we then proceeded to a different set of experiments to test other factors involved in propagation and scale up. Based on our findings in this study, we could have even chosen to omit the addition of MES altogether, by subculturing explants before the medium pH falls too low. In that case, we could reduce cost and perhaps establish a more economic micropropagation protocol for the industry.

2. The DCR medium itself is a modified version of the MS basal salts recipe developed for Douglas-fir by Gupta and Durzan (1985 and 1987). We further modified the DCR medium and classified it as mDCR, which is still based on MS. A comparison chart of the components between DCR vs. mDCR will be added in our revision.

**Competing Interests:** No competing interests were disclosed.

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