Adventitious shoot organogenesis from leaf and petiole explants of European hazelnut

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Abstract

Adventitious shoot organogenesis and somatic embryogenesis are the basis for implementing new genetic variability and biotechnological approaches in woody species, particularly if mature tissues from valuable cultivars are used. To date, these technologies are only applied to few tree species due to the absence of efficient regeneration protocols. In hazelnut, adventitious shoot organogenesis and somatic embryogenesis have only been carried out with zygotic embryo tissues up to now. Here we report plant regeneration from explants of somatic origin by using *in vitro* rejuvenated mature tissues (leaves, petioles and stipules). A histological analysis carried out on calli grown on various media, showed significant evidence of shoot regeneration, as proved by the presence of vascular elements such as tracheids with annular or helical secondary wall thickening. Subsequently, the optimization of the regeneration protocol performed by pre-treating the explants with antibiotics (carbenicillin, vancomycin, and cefotaxime) as molecules with auxin-like effects enabled us to achieve shoot organogenesis in hazelnut. The organogenic competence strictly depended on the explants and antibiotics used in the experiments. Following an antibiotic pre-treatment of cv Tonda Gentile Romana explants in the proliferation stage, organogenic responses (frequencies of 40%) were obtained. According to the results obtained, the best protocol for inducing shoot organogenesis in hazelnut should include the use of explants (leaves and petioles) conditioned in a double-liquid layer of cefotaxime 1000 mg L^{-1} added to the proliferation medium, cultured on the induction medium, consisting in solid MS medium supplemented with 3% sucrose, 6-benzylaminopurine 1 mg L^{-1}, indole-3-butyric acid 1 mg L^{-1} and kinetin 2 mg L^{-1}, and then by sub-culturing the newly-formed calli to regeneration medium, consisting of half-strength solid MS medium with sucrose (30 g L^{-1}) and 6-benzylaminopurine (0.5 mg L^{-1}).

Keywords: *Corylus avellana* L., shoot regeneration, rejuvenation, histological analysis, antibiotics

Introduction

The European hazelnut (*Corylus avellana* L.) is one of the most important tree nut crops in terms of worldwide production. Hazelnut is a monoecious, dichogamous, wind-pollinated plant and has a sporophytic incompatibility controlled by a single *S-locus* with multiple alleles (Mehlenbacher 1997). Today, hazelnut cultivation is steadily increasing in terms of the number of cultivation areas, especially in Countries where the availability of large agricultural lands and low labour costs allow hazelnut to be one of the most profitable tree crops.

The various varieties of hazelnut have been selected over time from natural wild populations in Europe which later spread to other parts of the world. Nowadays there are about 400 local varieties even if world production is only obtained from 20 cultivars (Cristofori et al. 2008).

For hazelnut micropropagation, many basal salts have been studied compared the most commonly used and recently Hand et al. (2014) and Hand and Reed (2014) studied the required mineral nutrient concentrations for micropropagation of hazelnut cultivar, concluding that the requirements are strongly cultivar-dependent.

The objectives of the genetic improvement have been clearly defined in order to satisfy the hazelnut demand, yet there is little information concerning the genetic parameters for the most targeted traits; although breeding programs are developing new cultivars for kernel and in-shell markets, much research is still required on this topic (Mehlenbacher 2009).

Due to the lack of information on the genetic basis of target traits, parents and their offspring are crossed and self-fertilization is not included due to self-incompatibility. Cross breeding is very complicated since the seedlings only start blooming after 5 years, as this species is characterized by a long juvenile period. However, by means of conventional breeding, some genotypes with improved traits have been released over the last few years. As alternatives to conventional methods of genetic improvement (clonal selection, crossing and selection and hybridization with other species with different gene pool), unconventional methods, such as selection of mutants from *in vitro* somaclonal variation, or induced by physical and chemical agents, may be quicker methods for obtaining new genotypes. To this aim, more efficient protocols of hazelnut *in vitro* regeneration are required.

Adventitious shoot organogenesis and somatic embryogenesis could be the key procedures for rapid genetic improvement in hazelnut. Up to now, neither somatic embryogenesis nor adventitious shoot regeneration have been achieved from *in vitro* rejuvenated mature tissues. The first report on hazelnut *in vitro* culture described embryoid induction, obtained by carrying out a callus culture on

mature zygotic embryos, cultured over two years, which are able to differentiate secondary somatic embryogenesis (Radojevic et al. 1975). Small, rounded nodules appeared on the callus surface which produced pro-embryonic structures when they were cultured in a modified MS medium supplemented with kinetin (kin) and 2,4-dichlorophenoxyacetic acid (2,4-D) for several months. Furthermore, the authors observed that the growth regulator 2,4-D did not inhibit the embryoids induction, whilst it arrested their further development into plantlets (Radojevic et al. 1975).

Rodriguez et al. (1984) reported that the culture of cotyledonary node segments in the presence of Indole-3-butyric acid (IBA) (1 mg L^{-1}) plus 6-benzylaminopurine (BAP) (0.1 mg L^{-1}) or BAP (1 mg L^{-1}) and IBA (0.1 mg L^{-1}), resulted in somatic embryogenesis induction. Subsequent proliferation was successfully maintained for five subcultures in the presence of BAP (0.1 mg L^{-1}). Following this procedure, an efficient regeneration percentage (55%) was reached and maintained in 60% of the explants. Over the last few years many studies on adventitious shoot regeneration and somatic embryogenesis in hazelnut have been published (Ayun et al. 2009), but all of them were focused on the use of zygotic tissues. Set up a regeneration protocol for an elite cultivar could be very interesting since *in vitro* propagated and "rejuvenated" plant of mature origin may retain their physiologic maturity during *in vitro*-culture (Nas et al. 2003).

It is a well-known fact that some antibiotics can affect morphogenesis. The first observations were made in *Agrobacterium*-mediated transformation experiments, where antibiotics, usually β-lactams, are added to the regeneration media with the aim of eliminating the bacteria used for transformation, since β-lactams inhibit peptidoglycan cross-linking during bacterium cell wall synthesis. Since plants do not synthesize the peptidoglycans, they are not known to affect plant growth, yet some papers demonstrate otherwise (Bosela 2009). It has been proved that carbenicillin often causes an auxin-like effect on callus growth, while cefotaxime stimulates adventitious morphogenesis (Bosela et al. 2009). This paper reports shoot regeneration starting from *in vitro* rejuvenated mature tissues of a hazelnut cultivar, by pre-treating the shoots with antibiotics.

Material and methods

Plant material

The experiments were carried out with explants obtained from two-year-old axenic cultures of cv Tonda Gentile Romana hazelnut, grown on Hazelnut medium (Bacchetta et al. 2008), supplemented with BAP (1 mg L^{-1}), zeatin (0.5 mg L^{-1}), Gibberellic acid (0.2 mg L^{-1}), and kept in a growth

chamber at +24±1°C with a 16-h photoperiod of 40 μmol m^{-2} s^{-1} provided by fluorescent lamps, subcultured every 25 days in 500 ml glass jars containing 100 mL of solid medium and 15 explants.

Experiment I: Adventitious shoot induction

Leaves, petioles, internodes and stipules collected at the end of the shoot proliferation cycle were used for carrying out regeneration experiments. These explants were cultured on MS medium, supplemented with sucrose (30 g L^{-1}) and plant agar (0.55%); the pH of the medium was adjusted to 5.8 before autoclaving for 20 min at 121°C. Following sterilization, the induction medium A (BAP 2 mg L^{-1}+NAA 0.5 mg L^{-1}), medium B (BAP 1 mg L^{-1}+IBA 0.01 mg L^{-1}+ Kin 2 mg L^{-1}) and medium C (BAP 1 mg L^{-1}+IBA 2 mg L^{-1}+ Kin 2 mg L^{-1}) were prepared, and 25 mL were poured into each Petri dish (ø 96 mm) containing ten explants. At least 30 explants for each kind of explant and for each induction media were tested. The cultures were kept in the dark for a week and then transferred to 16-hour light photoperiods in the same environmental conditions described above. After four weeks, the neo-formed calli were transferred to a new medium consisting of half-strength MS medium, supplemented with sucrose (30 g L^{-1}), plant agar (0.55%) and BAP (0.5 mg L^{-1}), and the culture were placed under light photoperiod.

Samples of the newly-formed calli were fixed in absolute ethanol-acetic acid (3:1 v/v) for 24-48 h, and were subsequently dehydrated in an ethanol series, cleared in xylene and embedded in Paraplast (Sigma). Each callus was sectioned at 12 μm thickness with a rotatory microtome. The sections were stained with 0.1% aniline blue in 0.1 M phosphate buffer, pH 12.4 (O'Brien and McCully 1981) and observed under an epifluorescence microscope (DMRB, Leica).

Experiment II: Regeneration by using explants collected from shoots proliferated on double-liquid layer with antibiotics

Following the results of the previous experiment on callus morphogenesis, three antibiotics (carbenicillin, vancomycin and cefotaxime), were tested individually with the aim of inducing adventitious morphogenesis. The antibiotics were dissolved in distilled water and a double-phase (solid/liquid) propagation system was established. Fifteen millilitres of antibiotic aqueous solutions at a concentration of 1000 mg L^{-1} were added to the solid medium after fifteen days in culture, while 15 mL of distilled water were added to the control. Fifteen days later, shoot growth (shoot height, number of nodes per explants and internode length) was recorded. The leaves, petioles, internodes and stipules from these shoots were collected and cultured in adventitious shoot induction medium C and the same procedure as the first experiment was used. Three weeks later

when the explants had formed abundant calli, they were transferred to half-strength MS medium, supplemented with sucrose (30 g L^{-1}), plant agar (0.55%) and BAP (0.5 mg L^{-1}), under the same environmental conditions as described above.

The regenerated shoots were placed on proliferation medium with the aim of obtaining new shoots for the subsequent rooting phase. Rooting was carried out on half-strength MS medium supplemented with sucrose (2%) and indol-3-butyric acid (1 mgL^{-1}) in 200 mL jars with 50mL medium. The jars were kept in the dark for a week and were then placed in the light. A month later when the roots start to elongate, the plantlets were transplanted into Jiffy pots for the hardening phase.

Statistical analyses

The data were subjected to the analysis of variance (ANOVA). The means were separated according to Duncan's test ($P \leq 0.05$), using R software package (http://cran.rpoject.org). Regeneration frequency was expressed in percentages.

Results and Discussion

Experiment I: Adventitious shoot induction

The explants forming a callus first showed a yellowing effect on the excised portion of the explants, petioles and leaf midribs. The stipules turned brown in a few days and produced small calli in few cases.

The callus formation rate varied according to the induction medium. On induction medium A, an early callus formation was observed, but the nodules, a putative sign of differentiation, only formed on the induction medium C

When the explants with callus were transferred on half-strength MS medium, supplemented with sucrose (30 g L^{-1}), plant agar (0.55%) and BAP (0.5 mg L^{-1}), spots of red pigments appeared on the callus surface probably due to an increase of anthocyanin biosynthesis, which is usually stimulated by the exogenous and endogenous cytokinins as reported in other species (Paulraj et al. 2014; Mulinacci et al. 2008; Crouch et al. 1993). The presence of the red spots might be related to morphogenesis as demonstrated by Crouch et al. (1993) in *Oxalis reclinata* where the onset of organogenesis during callus development was accompanied by a red pigmentation of the callus. According to Paulraj et al (2014), the red colour guaranteed shoot formation by exogenous treatment with abscisic acid in *Arabidopsis thaliana* zygotic embryo explants. However there is no clear functional role of anthocyanins in shoot regeneration, although they may play photo-protectant

and anti-oxidant roles in protecting the explants from physical and environmental stresses (Crouch et al. 1993).

Histological observations carried out on thin sections of hazelnut calli from stipules did not reveal the presence of any differentiated cells and the calli appeared to be necrotic, except in a single case in which a tracheary element was recognizable, while many tracheary elements differentiated in calli obtained from leaves (Figures 1a, b, e, f), internodes (Figures 1 c, g) and petioles (Figures 1 d, h). In particular, tracheids with annular or helical secondary wall thickenings were visible, thus confirming the attempts of the explants to regenerate adventitious shoots. Tracheary elements generally appear in the early stage of *in vitro* organogenesis and precede the formation of meristemoids and shoot primordia (Gatz and Kowalski 2011).

Experiment II: Regeneration by using explants collected from shoots proliferated on double-liquid layer with antibiotics

As shown in Table 1, the explants growing in double phase medium enriched with carbenicillin and vancomycin showed an average shoot height significantly higher than the control; on the contrary cefotaxime drastically reduced shoot height of the shoots. The node number was strongly affected by the presence of cefotaxime in the double liquid-layer medium, compared to control shoots and the shoots growing in carbenicillin or vancomycin-enriched media respectively. The mean internode length proved to be higher in the presence of carbenicillin and vancomycin than cefotaxime or in the controls. Cefotaxime inhibited the growth of the young shoots thus causing browning and abnormal shoot morphology in a few days. Both carbenicillin and vancomycin significantly enhanced the growth of the shoots; in particular they were 2-fold higher than the control, while no significative differences were observed in the number of nodes (Figure 2a). Internode length was also affected as it was significantly longer than in the control. Furthermore, the plantlets treated with vancomycin and carbenicillin had larger leaves. Our observations are in line with many other authors who described the effects of different antibiotics on growth enhancement in various species (Yepes et al. 1994; Tamprasert and Reed 1997; Kaur et al. 2008; Mancharda et al. 2011).

The hazelnut explants collected from the shoots pre-treated by means of double liquid-layer of antibiotics showed a powerful regeneration capacity. Cefotaxime pre-treated explants were able to regenerate shoots from petioles (Figure 2b) and leaves (Figure 2c), carbenicillin pre-treated explants from leaves (Figure 2e) and vancomycin pre-treated explants from stipules only. Each callus generally regenerated a single adventitious shoot. No regeneration events were obtained in the controls. The role of antibiotics in stimulating adventitious shoot regeneration is still not clear;

although it had already been observed several years ago in other recalcitrant woody species such as the mature tissues of the olive cultivar (Rugini et al. 1995) and hybrid aspens (Bosela et al. 2009).

The newly-formed shoots were excised and transferred to the shoot proliferation medium, where they produced new shoots from their axillary buds (Figure 2d). Individual regenerated shoots were tested for their rooting-ability, which showed the emergence of the roots after 20 days in culture and a 60% plant survival similar to the control plants (data not shown).

On the basis of the results reported by Hand and Reed (2014) and Hand et al. (2014) about the importance of macro and micro nutrients on the quality of micropropagated hazelnuts, further investigations for optimizing the mineral compositions of the media employed in adventitious shoot organogenesis are needed.

Conclusions

Previous studies have shown that organogenesis and somatic embryogenesis in hazelnut were only possible from zygotic tissues, since the juvenility of the tissues normally plays a key role in facilitating the induction of these processes. This method was effective in inducing adventitious shoot regeneration from in vitro rejuvenated mature tissues of the cv Tonda Gentile Romana hazelnut which is an important commercial cultivar that was considered recalcitrant to shoot regeneration like other hazelnut cultivars. The key to success was the pre-treatment of the *in vitro* shoots before explant excision with antibiotics, and cefotaxime proved to be the most effective.

Antibiotics do not only trigger shoot organogenesis, they can also accelerate shoot growth, however research is still required in order to better understand their roles in these processes.

Histological analyses proved to be fundamental for detecting the presence of vascular elements in the early differentiation stage of the shoots. The availability of an efficient regeneration protocol beginning with the explants of the elite cultivar opens new horizons for the genetic improvement of the European hazelnut.

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Author contribution statement. Cristian Silvestri was responsible for conception and design of experiments, data analysis, drafting of the manuscript and edited the paper. Valerio Cristofori took care of study conception and design and drafting the manuscript. Marilena Ceccarelli conducted the histological analysis. Maria Eugenia Caceres conducted the histological analysis. Eddo Rugini took care of study conception and design and edited the manuscript. All authors read and approved the manuscript.

Conflict of interest. The authors declare that they have no conflict of interest.

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306 and effect of antibiotics on proliferation. Plant Growth Regul 15: 55-67.

307 Figure 1. Histological sections of calli derived from leaves (a, b, e, f), internodes (c, g), and petioles
308 (Fig. 4 d, h). Aniline blue staining. Bar = 40 µm.
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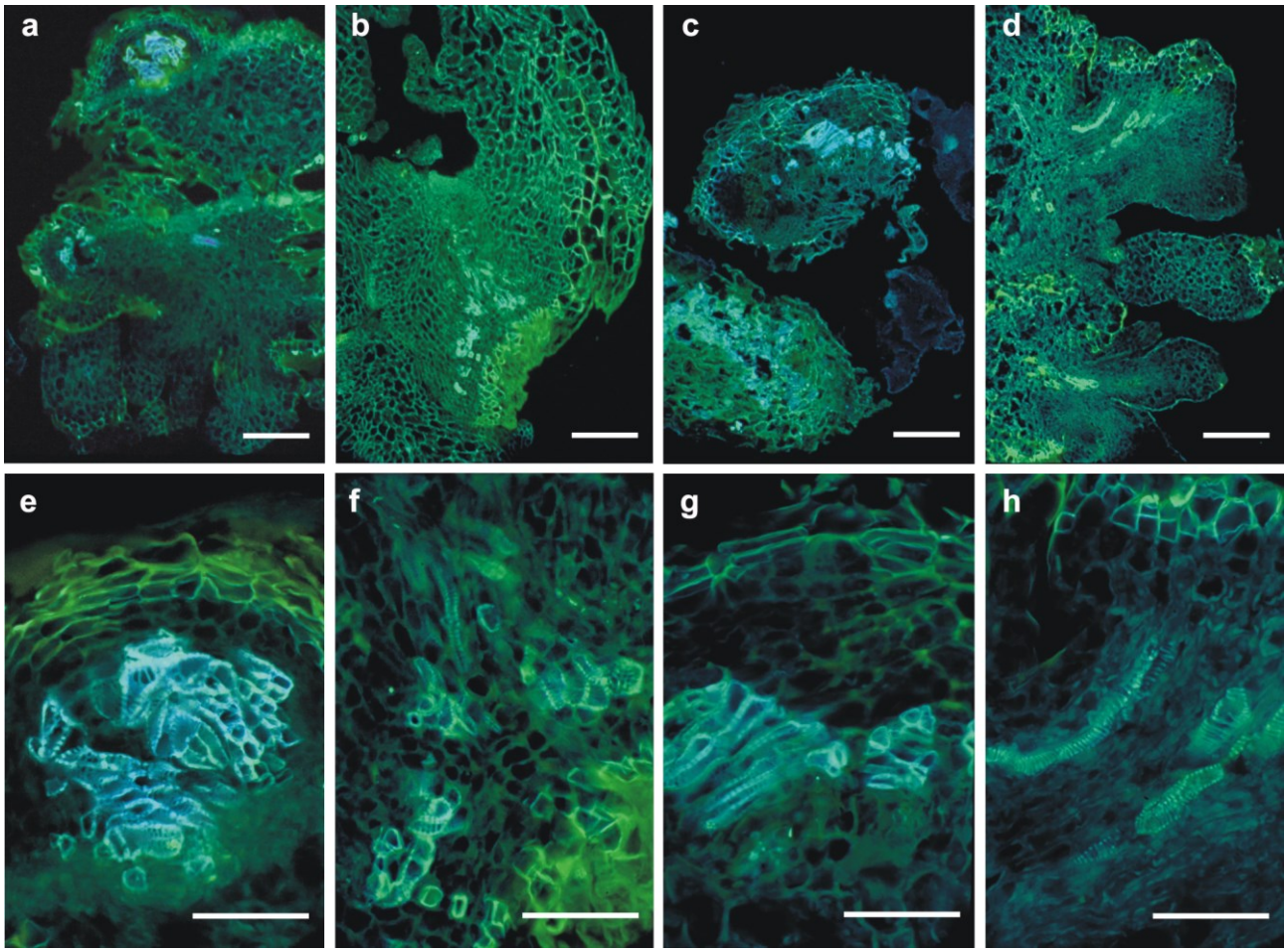

Figure 2. *In vitro* proliferation of hazelnut cv Tonda Gentile Romana on double-phase media. Proliferation rates obtained after pre-treatment with carbenicillin, vancomycin or cefotaxime were significantly higher than in the control (a). A small bud appeared on callus derived from petioles of a cefotaxime pre-treated shoot (b). Buds emerging from browned callus derived from leaves of a shoot pre-treated with cefotaxime (c). Regenerated explant excised and transferred in the proliferation medium(d). Cluster of buds arisen from a callus derived from leaves of a carbenicillin pre-treated shoot (e). Bar = 2 mm.

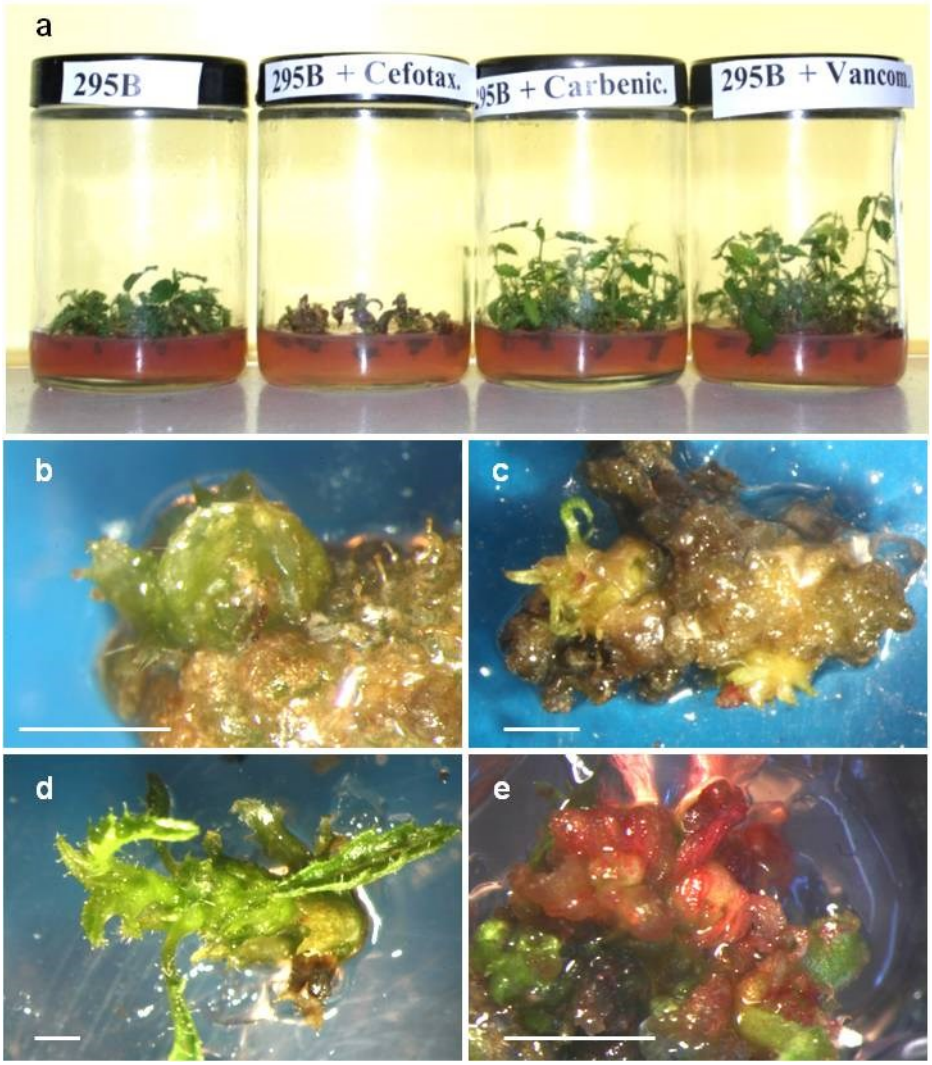

Table 1: Effects of antibiotics on vegetative parameters of hazelnut shoots in a proliferation medium with a double-liquid-layer system.

Antibiotics	Shoot height (mm)	No. of nodes per explants	Internode length (mm)
Water (Control)	26.2 **b**	4.2 **a**	6.2 **a**
Cefotaxime	11.0 **c**	2.0 **b**	5.5 **a**
Carbenicillin	57.8 **a**	5.1 **a**	11.3 **b**
Vancomycin	59.2 **a**	5.7 **a**	10.4 **b**

Data followed by the same letter are not significantly different (P≤0.05).

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