Third Symposium on Adventitious Root Formation. Veldhoven, Holanda

Adventitious rooting in microshoots of chestnut hybrid (*Castanea sativa* x *C. crenata*) in the presence of indole-3-butyric acid: The role of changes in endogenous indole-3-acetic acid, indole-3-acetylaspartic acid and indole-3-butyric acid levels

J.C. Gonçalves[1], G. Diogo[1], M.T. Coelho[1], N. Vidal[2] & S. Amâncio[3]

[1] *Lab. Biologia Vegetal, Escola Superior Agrária, 6000 C. Branco, Portugal*

[2] *Instituto de Investigaciones Agrobiológicas de Galicia, CSIC, Ap. 122, 15080 Santiago de Compostela, Spain*

[3] *Dep. Botânica e Engenharia Biológica, Instituto Sup. de Agronomia, 1399 Lisboa Codex, Portugal*

Correspondence author
José Carlos Gonçalves
Laboratório de Biologia Vegetal, Escola Superior Agrária, 6000 Castelo Branco, Portugal
Telephone: +351.272.339900
Fax: +351.272.339901
E-mail: jcgoncalves@esa.ipcb.pt

Abstract

Endogenous levels of IAA, IAAsp and IBA were quantified during the first 8 days of *in vitro* rooting of the chestnut hybrid, clone M3 by high performance liquid chromatography. Rooting was induced either by dipping the basal ends of the shoots into 1 gl^{-1} IBA solution for 1 minute or by subculturing the shoots on agar rooting medium with 3 mgl^{-1} IBA for 5 days. For root development the induced shoots were transferred to auxin-free agar medium. Auxins were measured in the apical and basal parts of the shoots by means of high performance liquid chromatography. Endogenous levels of IAA and IAAsp were higher in IBA-treated shoots than in control shoots. In extracts of the basal parts of the shoots, the concentration of free IAA showed a transient peak at day 2, in both root inductive methods, a subsequent gradual decrease for the remainder of the time course was observed. The concentration of IAAsp peaked at day 6 in extracts of the basal parts of shoots induced with 3 mgl^{-1} IBA for 5 days, whereas shoots induced by dipping showed an initial increase until day 2, and then remained stable. In extracts from basal shoot portions induced by dipping and by IBA in the medium, IBA concentration showed a transient peak at 24 h and 48 h, respectively, remaining stable after day 4 for the dipping method, whilst showing a significant drop between day 4 and 6 for the other induction treatment. All quantified auxins remained at a relatively low level, virtually constant, in extracts from apical shoot portions, as well as in extracts from control of non-rooting shoots.

Key words: auxins; chestnut; HPLC; IAA; IAAsp; IBA; rooting

Abbreviations: BHT=butylated hydroxytoluene; dw=dry weight; IAA=indole-3-acetic acid; IAAsp=indole-3-acetylaspartic acid; IBA=indole-3-butyric acid; MS=Murashige and Skoog mineral medium; NAA=naphthaleneacetic acid.

Introduction

The formation of adventitious roots depends upon a complex interaction between endogenous and environmental factors. While exogenous applied auxins are recognised as stimulators (Eliasson & Arebald 1984, Blazich 1988, Nordström & Eliasson 1991), the role of endogenous auxin in rhizogenesis is complex, and is still far from being fully understood (Moncousin, 1991; Blakesley 1994). The interdependent physiological phases of the rooting process are associated with changes in endogenous auxin concentrations (Moncousin et al. 1988, Hausman 1993). Blakesley (1994) and Gaspar et al. (1994) postulated that an early and transient increase of endogenous free auxin concentration occurs during the inductive phase of rooting. Furthermore Jarvis (1986) has proposed that an accumulation of IAA in the root-forming part of the cutting will function as the triggering factor for root initiation, whereas the later stage of primordial development is believed to be favoured by a lowered auxin content. Recently, Heloir et al. (1996) reported that the IAA content during rooting of *in vitro*-propagated walnut shoots showed a transient peak at 48 - 60 h and then exhibited a relatively low concentration for the remainder of the rooting phase. The fall in free IAA can be

attributed to conjugation, hydrolysis or to oxidative catabolism, which are important mechanisms in the regulation of free IAA levels in plant tissue (Bandurski 1980, Hand 1994).

In the case of chestnut cultured *in vitro*, IBA needs to be used in order to promote root formation (Vieitez et al. 1986, Gonçalves et al. 1998). The sequential anatomical changes during the rooting process reveal that the first cellular divisions occurred in some of the cambial derivative cells 24 h after auxin induction and a meristemoid became individualised by days 3-4, with the root primordia with a typical conical shape defined after 6-8 days; roots with organized tissue systems emerged from the stem 10-12 days after the root induction treatment (Gonçalves et al. 1998).

The main purpose of the present work was to monitor the concentration of endogenous IAA and one of its metabolites, IAA-aspartic acid, as well as the concentration of IBA, in *in vitro*-propagated chestnut shoots during the first 8 days and their role on adventitious root formation.

Materials and Methods

Plant Material and Culture Conditions

Stock shoot multiplication cultures of an hybrid adult clone of chestnut (*Castanea sativa* x *C. crenata*), clone M3, were multiplied as was previously described (Gonçalves et al. 1998). Axillary shoots, 3 to 4 cm in length were used for rooting. The basal medium used for chestnut rooting contained the nutrients of MS (1962) with macronutrients at half strength, with the exception of nitrates, which were reduced to quarter strength. For root induction, the basal ends of the shoots were dipped into 1 gl^{-1} IBA solution for 1 min or planted for 5 days in a 3 mgl^{-1} IBA agar medium. In order to allow root development, the induced shoots were transferred to basal auxin-free agar medium containing 6 gl^{-1} activated charcoal (Merck, 2184). During the rooting process, the shoots were kept in the growth chamber at a day/night temperature of 25/20 ºC with a 16 h photoperiod and a photosynthetic photon flux density of 45±5 μmol m^{-2} s^{-1} provided by cool-white fluorescent lamps. Almost 100% rooting of isolated shoots was achieved (Gonçalves et al. 1998). Non-rooting shoots cultured on basal rooting medium in the absence of IBA were used as controls.

Extraction and Quantification of Auxins

Apical and basal parts of equal length (1.5 to 2.0 cm) of the shoots, separately and without leaves, were used for the extraction and analysis of auxins. The shoots were collected daily until day 8, immediately frozen in liquid nitrogen and stored at -86 ºC. The samples were freeze-dried and the lyophilised powder was extracted with the method described by Nordström & Eliasson (1991) and Nordström et al. (1991). Briefly, 100 mg of the lyophilised powder of each part of the shoots was homogenised in 15 ml of 5 mM K-phosphate buffer, pH 6.5, containing BHT as antioxidant and NAA as internal standard. After 1 h in darkness the extract was filtered through a glass-fibre filter which was then rinsed with 5 ml of the extraction buffer and divided into two 10 ml portions. Each portion was first run through a Chromabond C18 column, 100 mg, activated with 2 ml of ethanol and conditioned with 2 ml of double distilled water and extraction buffer at a pH 6.5, and then washed with 5 ml of 5 mM K-phosphate buffer, pH 6.5. The eluate, containing the putative IAA, IAAsp and IBA, was acidified to pH 2.5 with 2.5 M phosphoric acid and applied to two C18 columns activated and conditioned to pH 2.5. The columns were washed with 2 ml distilled water, 2 ml acidic ethanol (ethanol:acetic acid:water, 20:2:78, v:v) and again with 2 ml distilled water. As the acidic ethanol fraction contained a great part of IAAsp it was recovered, the solvents were

evaporated in a Speed-Vac system at 30 ºC and the residue dissolved in 1.2 ml 80% methanol. Auxins were eluted from each of the two C18 columns (pH 2.5) with 100 µl aliquot of 100% methanol and 500 µl aliquot of 80% methanol.

The combined methanol extracts (1.2 ml) were filtered through a 0.22 µm nylon filter, and 30 µl of each sample was injected into a Waters HPLC, model 590, with a fluorescence detector, Waters 470 (excitation 292 ηm, emission 360 ηm), and a fully automated injection Waters 717 plus Autosampler system. The HPLC column used was a Hypersil ODS, 6 cm long, 3 µm particle size, solvent and column at 20 ºC, flow rate 1 ml min^{-1}. The mobile phase was 84% acetonitrile/glacial acetic acid/ water (10/2/88, v/v) and 16% acetonitrile for the IAA and IBA fraction, and 95% and 5%, respectively, for the IAAsp fraction. The system was operated isocratically. Under these experimental conditions, IAAsp eluted after 1.68 min, IAA after 3.38 min and IBA after 9.55 min. Auxins were quantified by computing the peak area using Millennium™ Chromatography Manager 2010 software. Fractions were collected and analysed through GC-MS, to confirm the identity of IAA.

The 100 mg of the lyophilised powder used for each sample extraction were obtained from 24 apical or basal parts of the shoots. Mean values and standard errors were calculated from the pooled values of two replicates from three separate experiments and were expressed by ηmol g^{-1} dw.

Results and Discussion

Over the 8-day culture period the concentration of free IAA in non-rooting control shoots remained constant, at very low concentrations, either on the basal or apical part of the shoots (Fig. 1A and B). The internal IAA concentration in the treated shoot bases increased significantly up a transient peak during the first 2 days, decreasing thereafter. However, by day 8 the values were significantly higher than in control shoots (13.64 ηmol g^{-1} dw for shoots treated by dipping, 6.18 ηmol g^{-1} dw for shoots treated with IBA on the medium, compared with 0.82 ηmol g^{-1} dw for control shoots) (Fig. 1A). In the apical parts of the IBA treated shoots (Fig. 1B), the maximum concentration of IAA was observed on day 1, with 7.1 ηmol g^{-1} dw, this being almost one fifth of the values achieved on the basal shoot parts at day two (Fig. 1A). An increase in the concentration of free IAA was also been observed in vine cuttings (Moncousin et al. 1988), during *in vitro* rooting of wild cherry (Label, et al., 1989) and walnut (Heloir et al. 1996) after IBA treatment. This increasing IAA concentration in chestnut seems to be an important step, one that is necessary in order to achieve the effective induction phase of rooting with the correspondent meristemoid differentiation which occurs between days 3-4 (Gonçalves et al. 1998). Subsequent IAA decrease should enable the evolution of the meristemoids into root primordia, which occurs between days 6-8 (Gonçalves et al. 1998). These variations in the concentration of IAA correspond to an inverse variation in peroxidase activity as occurs in chestnut (Gonçalves et al. 1998), oak (San-José et al. 1992), walnut (Ripetti et al. 1994) and vine (Gaspar et al. 1994).

The IAAsp concentration remained stable either in the basal or in the apical portions in non-rooting control shoots (Fig. 2A and B). In IBA treated shoots the IAAsp concentration increased, especially in those treated with IBA in the rooting medium (Fig. 2A) which may indicate that conversion to IAAsp could be the predominant metabolic route for regulation of the IAA content. In a similar experiment, Heloir et al. (1996) reported, however, that IAAsp concentration in the basal portions of rooting walnut shoots peaked about 36 h earlier than the

free IAA (12 h *versus* 48 h). In comparison to our results, this difference may be due to genetic characteristics or may reflect differences in the time of IAA conjugation. The potential role of IAA conjugates in the control of adventitious root initiation has been discussed by several authors (Nordström & Eliasson 1991, Blakesley 1994). Nevertheless, IAAsp applied to the rooting solution did not stimulate rooting (Nordström et al. 1991), which supports the assumption that once endogenous IAA is conjugated to IAAsp it does not stimulate adventitious root initiation.

In basal portions of the dip-treated shoots, the changes in the concentrations of IBA, closely paralleled the changes in IAA. In fact, the basal portions of the shoots cultured in IBA rooting medium showed a pronounced increase until day 2 (Fig. 3A), maintaining high levels until day 4 of culture on IBA rooting medium; a marked decrease was observed between days 4 and 6, when the shoots were transferred to an IBA free medium. The shoots treated by dipping peaked 24 h earlier than the shoots cultured in IBA rooting medium. Once again the apical portions of the shoots showed significantly lower concentrations when compared to the basal portions (Fig. 3B). As expected, in the control shoots the IBA concentration remained low throughout the experiment (Fig. 3A and B).

The hormonal quantification presented on this work shows that exogenous IBA led to high IAA concentration in the basal parts of the shoots, where roots were formed. The promotive effect of IBA on root neoformation could be explained either as an effect on its own or by an influence on internal IAA concentrations. The first statement was reported by Nordström et al. (1991) during adventitious root formation in pea cuttings, and the second was suggested by Dunberg et al. (1981) in *Pinus sylvestris* and Epstein & Lavee (1984) in grapevine and olive. The increase in IAA concentration may be due to a fast transformation of IBA into IAA, as has been reported by other authors in other species (Epstein & Lavee 1984, Van der Kriken et al. 1992, Epstein & Ludwig-Müller 1993) rather than from the hydrolysis of auxin conjugates (Bandursky 1980), since, in the same period, the IAAsp concentration showed a gradual increase (Fig. 2A). However, this remains to be confirmed by the use of labelled compounds. The subsequent decrease of IAA is possible due to the transformation of free IAA into conjugated IAAsp, once it is well known that plant tissues contained most of their IAA as derivatives, either esterified or as a peptide which were found in rich abundance in different species (Bandurski & Schulze, 1977; Domagalski et al., 1987).

The patterns of endogenous variations of IAA and IAAsp between the two root inducing systems were quite similar, which may indicate that these two methods do have similar results on rooting performance, as has been shown in a previous work (Gonçalves et al. 1998).

Acknowledgements

Thanks are given to Dr. A. Ballester for his comments on critical reading of manuscript. Technical assistance of Conceição Vitorino and Raquel Sabel is gratefully acknowledged. This research was partially supported by JNICT under PRAXIS XXI/2/2.1/BIO/1064/95 and by INIA by PAMAF 6127 projects.

References

Bandurski RS (1980) Homeostatic control of concentrations of indole-3-acetic acid. In: Skoog F (ed) Plant growth substances (pp 37-49). Springer-Verlag, Berlin

Bandurski RS & Schulze A (1977) Concentration of indole-3-acetic acid and its derivatives in plants. Plant Physiol 60: 211-213

Blakesley D (1994) Auxin metabolism and adventitious root initiation. In: Davies TD, Haissig BE (eds) Biology of adventitious root formation (pp 143-154). Plenum Press, New York

Blazich FA (1988) Chemical and formulations used to promote adventitious rooting. In: Davies TM, Haissig BE, Sankhla N (eds) Adventitious root formation in cuttings (pp 132-149). Dioscorides Press, Portland, OR, USA

Domagalski W, Schulze A & Bandurski RS (1987) Isolation and characterisation of esters of indole-3-acetic acid from the liquid endosperm o the horse chestnut (*Aesculus* species). Plant Physiol 84: 1107-1113

Dunberg A, Hsihan S & Sandberg G (1981) Auxin dynamics and the rooting of cuttings of *Pinus sylvestris*. Plant Physiol 67:S-5 (Abstr nº 19)

Eliasson L & Arebald K (1984) Auxin effects on rooting of pea cuttings. Physiol Plant 61: 293-297

Epstein E & Lavee S (1984) Conversion of indole-3-butyric acid to indole-3-acetic acid by cuttings of grapevine (*Vitis vinifera*) and olive (*Olea europea*). Plant Cell Physiol 25: 697-703

Epstein E & Ludwig-Müller J (1993) Indole-3-butyric acid in plants: occurrence, synthesis, metabolism and transport. Physiol Plant 88: 382-389

Gaspar T, Kevers C, Hausman JF & Ripetti V (1994) Peroxidase activity and endogenous free auxin during adventitious root formation. In: Lumdsen PJ, Nicholas JR, Davies WJ (eds) Physiology, growth and development of plants in culture (pp 289-298). Kluwer Acad Pub, Dordrecht

Gonçalves JC, Diogo G & Amâncio S (1998) *In vitro* propagation of chestnut (*Castanea sativa* x *C. crenata*): Effects of rooting treatments on plant survival, peroxidase activity and anatomical changes during adventitious root formation. Sci Hortic 72: 265-275

Hand P (1994) Biochemical and molecular markers of cellular competence for adventitious rooting. In: Davies TD, Haissig BE (eds) Biology of adventitious root formation (pp 111-121). Plenum Press, New York

Hausman JF (1993) Changes in peroxidase activity, auxin level and ethylene production during root formation by poplar shoots raised *in vitro*. Plant Growth Regul 13: 262-268

Heloir M-C, Kevers C, Hausman J-F & Gaspar T (1996) Changes in the concentrations of auxins and polyamines during rooting of *in-vitro*-propagated walnut shoots. Tree Physiol 16: 515-519

Jarvis BC (1986) Endogenous control of adventitious rooting in non-woody cuttings. In: Jackson MB (ed) New root formation in plants and cuttings (pp 191-222). Martinus Nijhoff Pub, Dordrecht

Label PH, Sotta B & Miginiac E (1989) Endogenous levels of abscisic acid and indole-3-acetic acid during *in vitro* rooting of wild cherry explants produced by micropropagation. Plant Growth Regulation 8: 325-333

Moncousin C (1991) Rooting *in vitro* cuttings. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, 17. High-tech and micropropagation (pp 213-261). Springer-Verlag, Berlin

Moncousin C, Favre JM & Gaspar T (1988) Changes in peroxidase activity and endogenous IAA levels during adventitious root formation in vine cuttings. In: Kutacek M,

Bandursky RS, Krekule J (eds) Physiology and biochemistry of auxins in plants (pp 331-337). Academia Praha, Praha

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

Nordström A-C & Eliasson L (1991) Levels of endogenous indole-3-acetic acid and indole-3-acetylaspartic acid during adventitious root formation in pea cuttings. Physiol Plant 82: 599-605

Nordström A-C, Jacobs FA & Eliasson L (1991) Effect of exogenous indole-3-acetic acid and indole-3-butyric acid on internal levels of the respective auxins and their conjugation with aspartic acid during adventitious root formation in pea cuttings. Plant Physiol 96: 856-861

Ripetti V, Kevers C & Gaspar T (1994) Two successive media for rooting of walnut shoots *in vitro*. Changes in peroxidase activity and ethylene production. Adv Hortic Sci 8: 29-32

San-José MC, Vidal N & Ballester A (1992) Anatomical and biochemical changes during root formation in oak and apple shoots cultured *in vitro*. Agronomie 12: 767-774

Van der Kriken WM, Breteler H & Visser MHM (1992) The effect of conversion o indolbutyric acid into indolacetic acid on root formation on microcuttings o *Malus*. Plant Cell Physiol 33: 709-713

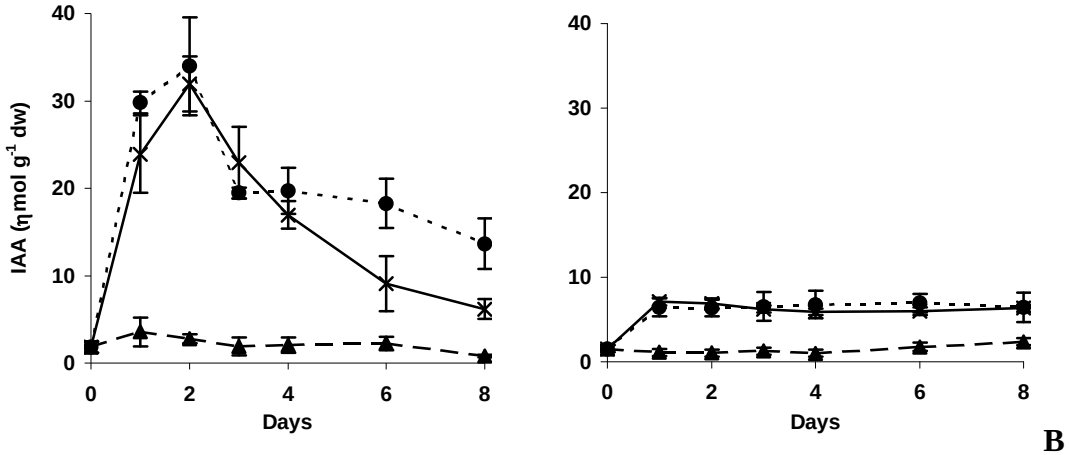
Vieitez AM, Vieitez ML & Vieitez E (1986) Chestnut (*Castanea* spp.). In: Bajaj YPS (ed) Biotechnology in Agriculture and Forestry (pp 393-414). Springer-Verlag, Berlin

LEGENDS

Fig. 1. Endogenous levels of IAA in chestnut shoots during the first 8 days of root formation. (A) basal portion of the shoots. (B) apical portions of the shoots. Horizontal bars showing SD (within symbols if not visible). (σ) control; (λ) treated shoots by dipping in 1 gl^{-1} IBA solution during 1 min; (6) treated shoots with 3 mgl^{-1} IBA during 5 days in rooting medium.

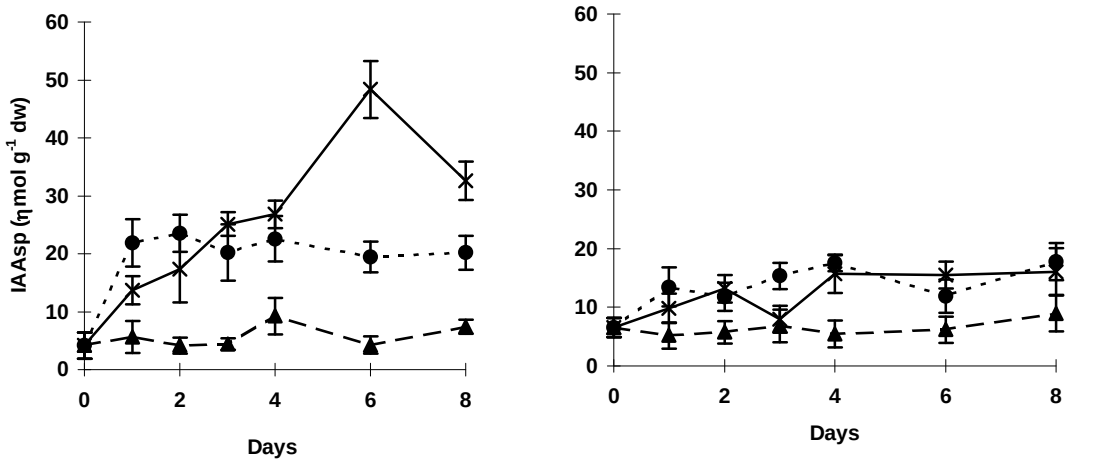
Fig. 2. Endogenous levels of IAAsp in chestnut shoots during the first 8 days of root formation. (A) basal portion of the shoots. (B) apical portions of the shoots. Horizontal bars showing SD (within symbols if not visible). (σ) control; (λ) treated shoots by dipping in 1 gl^{-1} IBA solution during 1 min; (6) treated shoots with 3 mgl^{-1} IBA during 5 days in rooting medium.

Fig. 3. Levels of IBA in chestnut shoots during the first 8 days of root formation . (A) basal portion of the shoots. (B) apical portions of the shoots. Horizontal bars showing SD (within symbols if not visible). (σ) control; (λ) treated shoots by dipping in 1 gl^{-1} IBA solution during 1 min; (6) treated shoots with 3 mgl^{-1} IBA during 5 days in rooting medium.



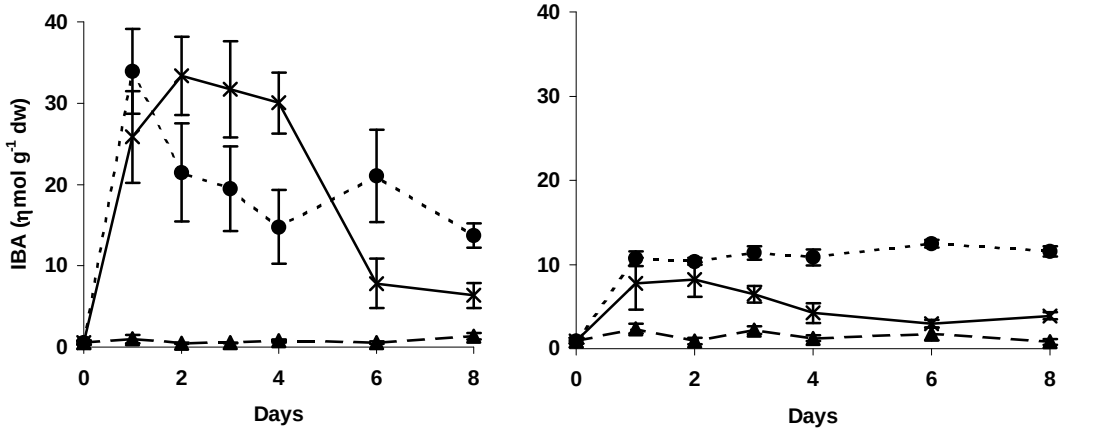
A **B**

Figure 1



A **B**

Figure 2



A **B**

Figure 3