# STUDENT PROJECT A comparison of natural and synthetic rooting hormones for vegetative propagation using *Saxegothaea conspicua* Lindl.

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## Abstract

This paper examines the effects of using natural and synthetic rooting hormones for propagation by cuttings. The study used *Saxegothaea conspicua* Lindl., a Near Threatened conifer native to Chile and Argentina. A trial was set up on 80 semi-ripe cuttings from a single clone of *S. conspicua*, applying both synthetic (IBA) and natural hormones derived from willow and lentils. The study evaluates the rooting success of different types of hormone using the measurements of the development of roots and their length at the end of the trial period. It also examines the level and type of auxins found in the lentil and willow rooting solutions, if present. After evaluation of the results and wider literature, the paper goes on to discuss the potential for use of this type of natural hormone in future, especially in the light of new European Union regulations which come into force in 2021.

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## Introduction

Plant hormones, or 'phytohormones', are defined as 'chemical messengers that control plant growth and development' (Hurný & Benkova, 2017). Rooting hormones can be either naturally occurring or synthetic, and they stimulate root growth in plants (Hopper, 2016).

Auxin (from the Greek *auxein*, to increase) was the first phytohormone to be discovered, and it has a principal role in the production of adventitious roots. Since they were first isolated in 1928 by F.W. Went, synthetic auxins have been further researched and used in plant propagation (Hopkins & Norman, 2009). Commercial rooting hormones are available in various formulations, such as liquids, powders and gels, and are widely used in commercial plant propagation due to their low cost and chemical stability. Indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA) auxins are the most used in vegetative propagation to increase rooting percentages, accelerate root formation and to increase the number and quality of roots (Blythe, 2013).

Conversely, there is a growing interest in natural rooting stimulants (Hartmann *et al*., 1997), and many organic growers do not want to use synthetic hormones (Hopper, 2016). Amateur gardeners or propagators in a non-commercial environment are more inclined towards natural rooting hormones

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as an alternative to synthetic hormones and claim that the same rooting effects can be achieved (Faber, 2016; Hopper, 2016). Natural alternatives include honey, willow, lentils, saliva, cinnamon, apple cider vinegar, coffee, soya and wheat (La Huertina De Toni, 2016; Hopper, 2016; Faber, 2016). Among these, this study focuses on the capacity of willow (*Salix* sp.) and lentils (*Lens* sp.) to stimulate rooting. *Salix* species contain root-promoting hormones such as IBA and salicylic acid (SA) (Diagneault & Chong, 1983; Dirr & Heuser, 2006; Deep Green Permaculture, 2009; La Huertina De Toni, 2016). For lentils, it is known that auxin can be found in actively growing organs such as germinating seeds (Dirr & Heuser, 2006).

Overall, there is a lack of robust scientific studies available on the internet on natural hormones, and the efficacy of this method should be demonstrated with objective research. In this context, this study compares synthetic and natural (plant-derived) rooting ability applied in cuttings of *Saxegothaea conspicua*, an endemic conifer from Chile and Argentina categorised by the International Union for Conservation of Nature (IUCN) as Near Threatened (Gardner *et al.*, 2006).

## Background information

### *Saxegothaea conspicua* (Podocarpaceae)

Monoecious evergreen tree to 25–30 m tall (Fig. 1) distributed through southern Argentina (Chubut, Neuquén, Rio Negro) and Chile (Aisén, Biobío, La Araucanía, Los Lagos, Maule). It mainly occurs in the wet Valdivian rainforests. Its altitudinal range is from near sea level to 1,000 m in the Andes, becoming scarce north of 38° due to the transition to a drier climate (Farjon, 2010).

*Saxegothaea conspicua* has been propagated from semi-ripe cuttings in the past using IBA rooting hormone at 2,000 ppm, and rooting takes two to three months with a 96 per cent success rate (Gardner *et al.*, 2006). Most conifer cuttings are taken in late autumn or early winter as exposure to the cold temperatures of that time of the year helps in rooting (Ruter, 2008).

### *The use of synthetic hormones: the role of auxin*

The principal auxin in plants is indole-3-acetic acid (IAA). This is a naturally occurring auxin in plants and is responsible for natural root stimulation. Yet IAA is not used in plant propagation because it is easily degraded by IAA oxydase, light and bacteria (Dirr & Heuser, 2006). Instead, all commercial preparations used in vegetative propagation are based on synthetic IBA, NAA and their derivatives. These synthetic chemicals are more stable and resistant to oxidation (Hopkins & Norman, 2009).

Auxin is not always the limiting chemical component in rooting; there are other naturally occurring substances that work in synergy with IAA. These are known as rooting cofactors or auxin synergists (Hartmann *et al*., 1997; Dirr & Heuser, 2006). A word of caution with auxin: if the applied concentration is high and exceeds the optimum, root formation will be inhibited (Schell, 2014).

### *The use of plant-derived hormones: non-chemical alternatives*

Plant-derived substances have been used for centuries (Hartmann *et al*., 1997). Many plant propagators are reluctant to use most commercial varieties of root stimulant because they include pesticides and synthetic hormones, and they are expensive (Faber, 2016). Instead, several natural rooting hormones are used as an alternative to

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**Fig. 1** *Saxegothaea conspicua*. Photo: Jeff Bisbee.

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synthetics. Sea kelp (Schell, 2014), honey, cinnamon, willow, lentils and saliva are the most prevalent among amateur propagators on the internet (Deep Green Permaculture, 2009; La Huertina De Toni, 2016; Faber, 2016; Hopper, 2016). These natural hormones achieve high success rates during cloning, although results will not be seen as quickly as when a synthetic rooting hormone is used (Hopper, 2016).

### *The use of lentils*

In the early days of cutting propagation, an ancient practice in the Middle East and Europe was to bury grain seeds (mainly cereals or legumes) into the split ends of cuttings. The physiological basis to support the rooting capacity of this method is that germinating seeds are a good source of auxin, as they contain high levels of IAA to encourage the rapid growth of the seedling when the seed germinates (Hartmann *et al*., 1997). Another chemical with auxin-like activity, 4-chloroindole-3-acetic acid, has been found in extracts of legume seeds (Hopkins & Norman, 2009).

### *The use of willow*

Willow extract is probably the best natural, organic rooting stimulator available and is also used for transplant shock (Tappinroots.com, 2016). There are many products available that use it as the main ingredient and these can naturally provide the same success rates as synthetic rooting hormone products (Hopper, 2016).

Kawase (1964) found strong root-promoting substances extracted from softwood cuttings of *Salix alba*. Diagneault & Chong (1983) stated that 'water soluble phenolic and indolic compounds are major root-promoting substances in willow extract'. SA is a natural antiseptic and has anti-fungal properties while IBA stimulates root growth, this being an example of synergy (Deep Green Permaculture, 2009). These hormones are found in high concentrations in the growing tips of willow branches (Bond, 2017).

## Materials & methods

A four-month trial was conducted in the Nursery at the Royal Botanic Garden Edinburgh (RBGE) between 4 December 2017 and 9 April 2018. The trial consisted of four treatments, with a total of eighty experimental units (Table 1).

Eighty semi-ripe heel cuttings 6–10 cm in length were collected on 4 December 2017 from wild-collected plants cultivated in the Nursery glasshouse. The mother plant was six years old with accession number 2013.0343 (Fig. 2). This represents a very rare example of known wild-origin material. This juvenile plant material came as a seedling from the wild in Chile. It was found on a fallen rotten tree in the region of Los Ríos, Valdivia (Walter & O'Neal, 1985–2020). Juvenile material roots better than mature plant material (Dirr & Heuser, 2006). The growing medium used was RBGE1, a peat-free, organically derived mix developed and supplied by Melcourt Industries, UK. Perlite and sand were also added to give a growing medium of RBGE1:perlite:sand at a ratio of 50:40:10. RBGE1 contains Growbark pine, a finely milled pine bark, Sylvafibre, a broken down

| Treatment | Hormone | Experimental unit |
|---|---|---|
| **C** | No hormones | 20 |
| **T1** | Synthetic: IBA at 2,000 ppm | 20 |
| **T2** | Natural: lentils | 20 |
| **T3** | Natural: willow | 20 |

**Table 1** Trial design.

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**Fig. 2** 2013.0343, mother plant in juvenile state. Photo: Isabel Cayon-Fernandez.

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woody substance, and compost made from green waste in the following proportions: 10:7:3.

Cuttings were grouped for space efficiency (Fig. 3). There were four cuttings per pot, each pot corresponding to a single treatment. Optipot Square 10k pots (with a diameter of 10 cm) were used, and had been previously washed and disinfected with water and detergent.

Control cuttings (C) were inserted with no hormones. For T1 (synthetic hormone) cuttings were dipped for two seconds in Clonex rooting hormone IBA 3.3 g/l. Both C and T1 pots were watered and placed on a heated bench. T2 and T3 (lentil and willow respectively) were treated differently. Cuttings were left imbibing lentil and willow solutions overnight (Fig. 4). After 24 hours, cuttings were potted up and placed on a heated bench, and then for one week were hand-watered when the growing medium was dry with 30 cl per pot of their respective solution water.

The whole experiment was placed inside an enclosed mist unit in a completely randomised design to avoid bias (Fig. 3) with a mist unit interval of fifteen seconds every two hours and a natural photoperiod. A thermometer was placed in one of the pots, with the basal temperature ranging between 17 and 20°C. Mist intervals were adjusted to 15 seconds every 1.5 hours during warm sunny spells (mainly in March).

## *Lentil solution preparation*

Methods followed La Huertina De Toni (2014). Brown lentils (*Lens culinaris*) were used for making the solution, with 200 g of lentils soaked overnight in a pyrex bowl in a 1:4 ratio (1 cup of lentils to 4 cups of water). This allowed seed imbibition which accelerated germination. The complete germination process took five days; after this time all the seeds developed a radicle. Germination took place in darkness, as the seeds were covered with a cloth, and at a room temperature of 16–18°C.

After five days, germinated lentils were blended with the remaining water. A sieve was used to separate the lentil mash from the resulting lentil solution. This concentrated solution was then poured into a two-litre plastic bottle using a funnel. A label with name, date and use-by date was placed on the bottle (Fig. 5). The solution could be used for up to 15 days if kept in the fridge. When applying the solution to the cuttings, a ratio of 1:10 (1 part of lentil solution to 10 parts of water) was used.

## *Willow solution preparation*

Methods followed La Huertina De Toni (2016). Soft tips (current season's growth) of *Salix babilonica* were collected on 27 November 2017 from four different trees located at Figgate Park (Edinburgh, UK); 400 mg of twigs were cut into 4–5 cm pieces and left in two litres of water for a duration of four days. The leaves had been previously removed. The mix was contained in a five-litre glass jar and covered with a cloth to provide darkness. After four days, the water containing the willow twigs was heated to just below boiling point for a duration of ten to fifteen minutes. This 'tea' was slow-cooked and care was taken that the water did not reach boiling point at any time. Once the solution had cooled (around two hours later), it was poured into a two-litre plastic bottle using a funnel. A label with name, date and use-by date was placed on the bottle (Fig. 5). The solution could be used for up to a month if kept in the fridge. There was no need to dilute this solution with water when applying it to the cuttings.

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**Fig. 3** Experiment layout. Photo: Isabel Cayon-Fernandez.

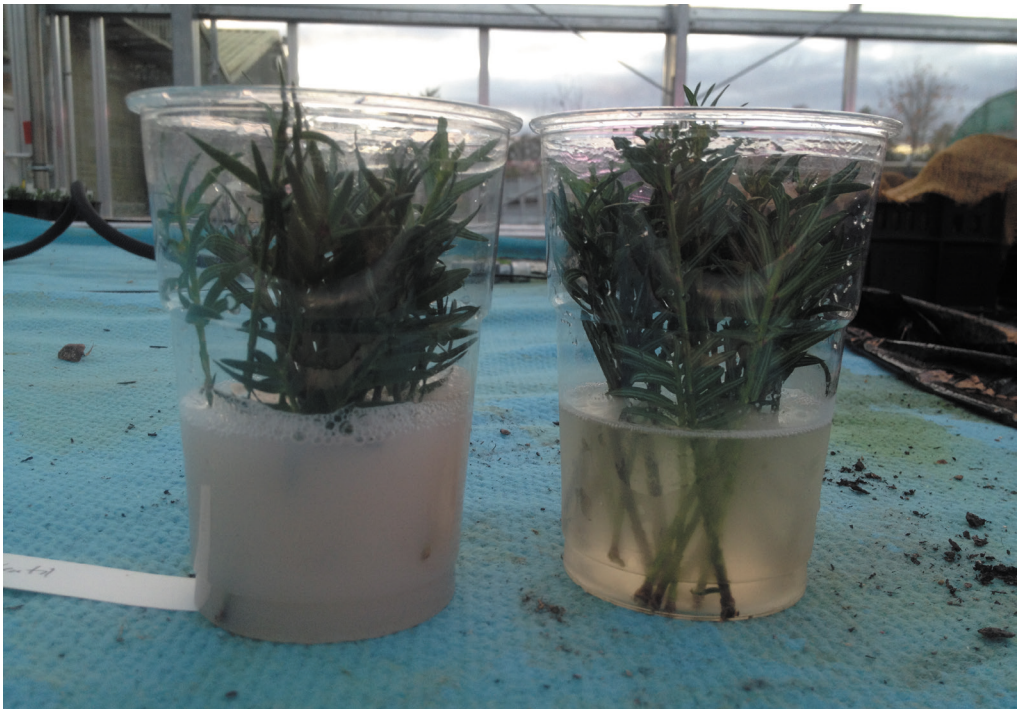

**Fig. 4** Lentil and willow imbibition. Photo: Isabel Cayon-Fernandez.

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**Fig. 5** Lentil and willow rooting hormones. Photo: Isabel Cayon-Fernandez.

## *Chromatography analysis*

Lentil and willow solutions were subject to chromatography analysis. An Exactive Orbitrap mass spectrometer, manufactured by ThermoFisher™, was used to analyse both homemade solutions (see Appendix B). After XCalibur 2.1.0 software analysis (ThermoFisher™ Scientific, Bremen, Germany), a resulting list of 50 top metabolites in both positive and negative mode was produced for both solutions (see Appendix A). Compound identification in both solutions allowed the detection of possible rooting factors upon completion of the trial (David Watson, pers. comm.).

## *Measurements: image analysis*

All eighty cuttings were imaged after the four-month experiment. Stem-cutting pictures were taken using a Xiaomi Mi A1 mobile phone with a 12MP 1.25µm f/2.2 lens at a resolution of 4,000 x 3,000 pixels. Images were saved as .jpeg format for further analysis. A portable A4 LED light table with scale was used as a background to minimise light variations between pictures and obtain an accurate image (Fig. 6). Pictures were used for measuring rooting quantitatively and qualitatively.

A morphometric analysis of each experimental unit was performed by importing each image into Fiji-ImageJ v.1.52a open source software. Each single image was scaled and applied with a colour threshold that had to be adjusted manually to maximise root and stem identification (Fig. 7). This non-destructive method of data gathering enabled the measurement of the root length, area and width traits for each cutting. Raw data were exported to Excel for subsequent data analysis.

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**Fig. 6** Root measurement with LED light table. Photo: Isabel Cayon-Fernandez.

## *Statistical analysis*

Differences between treatments were analysed using a one-way analysis of variance (ANOVA). Results of the test ($P < 0.05$) determined whether there was any statistically significant difference by comparing the means of the four treatments for length, width and area traits and, if there was, determined where exactly the difference was by using a Tukey post hoc test.

Qualitative data was analysed, creating a rooting index (Beyl & Trigiano, 2000). This index allowed each of the cuttings to be scored across the treatments by assigning a value from 1 to 5. For this trial, 'good' represents > 4 cm spread, 'medium' 1–4 cm spread and 'root initiation' up to 1 cm spread. A final score determined the overall treatment performance for rooting.

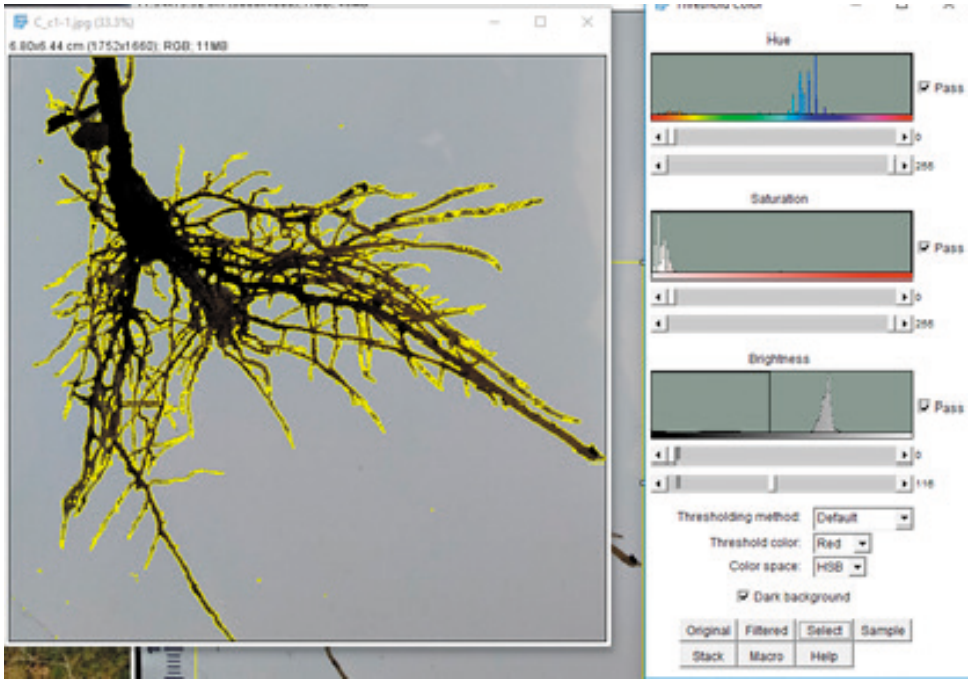


**Fig. 7** Example of ImageJ analysis. Photo: Isabel Cayon-Fernandez.

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## Results

### *Rooting index*

The rooting index (Table 2) scores all cuttings across treatments, giving a value from 1 to 5 (Beyl & Trigiano, 2000). After a period of four months, willow (T3) was the best treatment, scoring 4.55, meaning that the root system is good. IBA (T1) follows quite close behind, scoring 4.4 (good–medium), the control (C) with no hormones scored 4.15 (medium) and the treatment with the poorest score was lentils (T2) with 3.55 (root initiation–medium).

Table 2 also shows rooting percentages. In the control treatment 100 per cent rooted, followed by T1 and T3 with 95 per cent and T2 with 85 per cent.

### *Root area*

Table 3 shows that there was a statistically significant difference between treatments as determined by one-way ANOVA ($F = 7.97$, $P = 0.00$).

Fig. 8 shows difference of means with standard error of root area ($cm^2$) for all treatments. The effect of synthetic hormone IBA (T1) was higher than the other treatments for root area (4.90 $cm^2$). Natural hormone willow (T3) was higher than the control with no hormones (root area 3.98 $cm^2$ > 2.83 $cm^2$). The smallest root area was shown in the lentil treatment (T2) with only 1.30 $cm^2$.

Table 4 shows that there was a significant effect of synthetic hormones (T1) on root area at the $P < 0.05$ level for [$F$ (3, 76) = 7.97, $P = 0$]. Post hoc comparisons using the Tukey HSD test indicated that the mean score for T1 (mean = 4.90, SD = 3.20) was significantly different from C and T2. However, T2 and T3 did not significantly differ from the C treatment.

### *Root length*

Table 5 shows that there was a statistically significant difference between treatments as determined by one-way ANOVA ($F = 5.81$, $P = 0.00$).

Fig. 9 shows the difference of means and standard error of root length for all treatments.

| | | | Number of cuttings | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | **Replication** | **Cuttings set** | **Good (x5)** | **Medium (x4)** | **Root initiation (x3)** | **Alive (but no roots) (x2)** | **Dead (x1)** | **Sum of weights** | **Rooting index** | **% rooted** |
| Control | *Mean* | 20 | 8 | 7 | 5 | 0 | 0 | 83 | ***4.15*** | ***100*** |
| Treatment 1 | *Mean* | 20 | 12 | 5 | 2 | 1 | 0 | 88 | ***4.4*** | ***95*** |
| Treatment 2 | *Mean* | 20 | 4 | 7 | 6 | 2 | 1 | 71 | ***3.55*** | ***85*** |
| Treatment 3 | *Mean* | 20 | 13 | 6 | 0 | 1 | 0 | 91 | ***4.55*** | ***95*** |

**Table 2** Rooting index.

| Source of variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between groups | 144.4017 | 3 | 48.13392 | 7.970031 | 0.000109 | 2.724944 |
| Within groups | 458.9917 | 76 | 6.039364 | | | |
| Total | 603.3934 | 79 | | | | |

**Table 3** ANOVA results for root area ($cm^2$).

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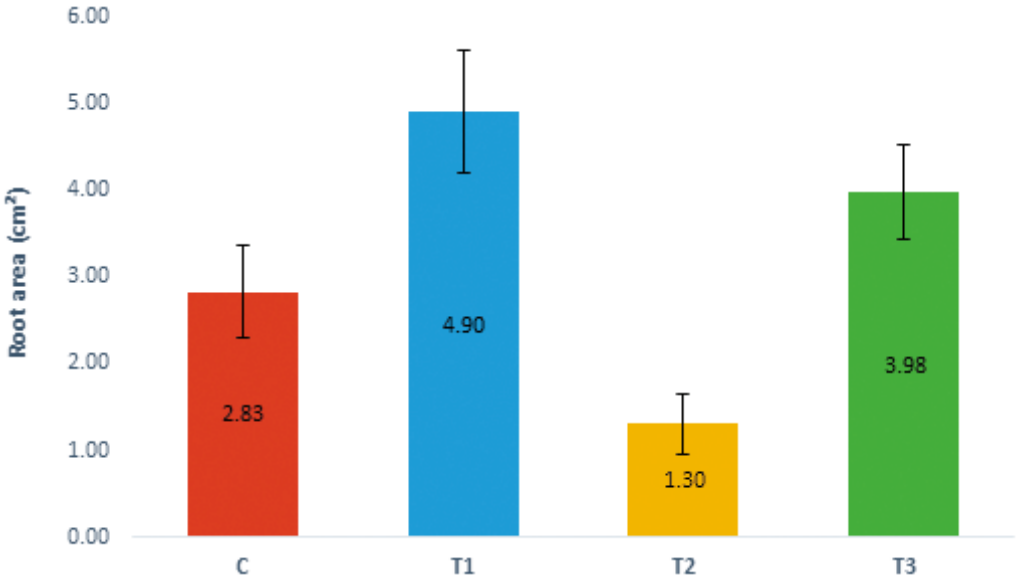


**Fig. 8** Difference of means with standard error of the mean for root area ($cm^2$) between treatments.

| Comparison | Absolute difference | Critical range |
|---|---|---|
| C to T1 | 2.073 | 1.820 |
| C to T2 | 1.5255 | 1.820 |
| C to T3 | 1.1455 | 1.820 |
| T1 to T2 | 3.5985 | 1.820 |
| T1 to T3 | 0.9275 | 1.820 |
| T2 to T3 | 2.671 | 1.820 |

**Table 4** Tukey test results for root area ($cm^2$).

T1 mean for root length is higher than for the other treatments (3.36 cm), followed by T3 with 3.01 cm. The control treatment had a mean root length of 2.44 cm. The lowest value is represented by T2 with a mean of 1.52 cm.

Table 6 shows that lentil treatment (T2) had a significant effect on root length at the $P < 0.05$ level for [$F$ (3, 76) = 5.81, $P = 0$]. Post hoc comparisons using the Tukey HSD test indicated that the mean score for T2 (mean = 1.52, SD = 1.50) was significantly different from T1 and T3.

| Source of variation | SS | df | MS | *F* | *P*-value | *F* crit |
|---|---|---|---|---|---|---|
| Between groups | 38.72122 | 3 | 12.90707 | 5.805081 | 0.001257 | 2.724944 |
| Within groups | 168.9792 | 76 | 2.22341 | | | |
| Total | 207.7004 | 79 | | | | |

**Table 5** ANOVA results for root length (cm).

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| Comparison | Absolute difference | Critical range |
|---|---|---|
| C to T1 | 0.9245 | 1.104 |
| C to T2 | 0.9155 | 1.104 |
| C to T3 | 0.5725 | 1.104 |
| T1 to T2 | 1.84 | 1.104 |
| T1 to T3 | 0.352 | 1.104 |
| T2 to T3 | 1.488 | 1.104 |

**Table 6** Tukey test results for length (cm).

## *Root width*

Table 7 shows that there was a statistically significant difference between treatments as determined by one-way ANOVA ($F = 4.14$, $P = 0.01$).

Fig. 10 shows the difference of means and standard error of width (cm) for all treatments. T3 mean for root width is higher than the other treatments (0.10 cm), followed by T1 with 0.09 cm. The control treatment has a mean width of 0.08 cm and the smallest value is represented by T2 with a mean of 0.07 cm.

Table 8 shows that willow treatment (T3) had a significant effect on root width at the $P < 0.05$ level for [$F$ (3, 76) = 4.14, $P = 0$]. Post hoc comparisons using the Tukey HSD test indicated that the mean score for T3 (mean = 0.10, SD = 0.03) was significantly different from C and T2. However, T3 did not significantly differ from the T1 treatment.

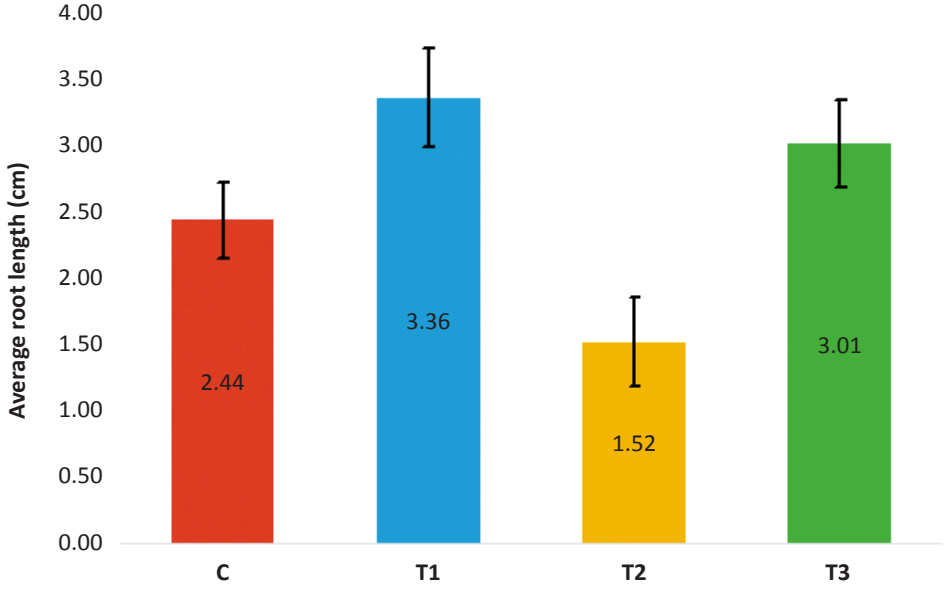


**Fig. 9** Difference of means with standard error of the mean for length (cm) between treatments.

| Source of variation | SS | df | MS | *F* | *P*-value | *F* crit |
|---|---|---|---|---|---|---|
| Between groups | 0.006745 | 3 | 0.002248 | 4.142384 | 0.008952 | 2.724944 |
| Within groups | 0.04125 | 76 | 0.000543 | | | |
| Total | 0.047995 | 79 | | | | |

**Table 7** ANOVA results for root width (cm).

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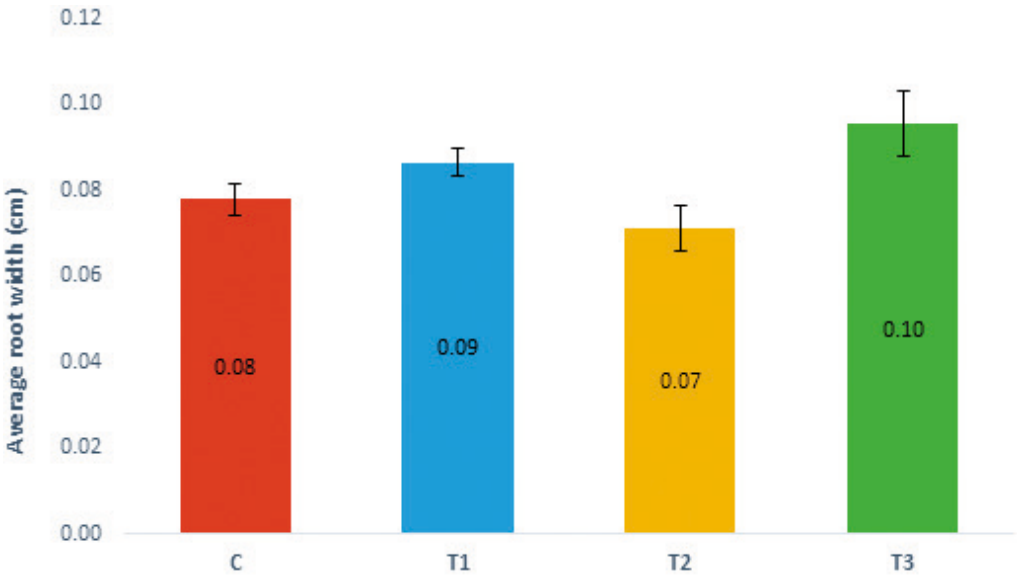


**Fig. 10** Difference of means with standard error of the mean for root width (cm) between treatments.

| Comparison | Absolute difference | Critical range |
|---|---|---|
| C to T1 | 0.0085 | 0.0173 |
| C to T2 | 0.007 | 0.0173 |
| C to T3 | 0.0175 | 0.0173 |
| T1 to T2 | 0.0155 | 0.0173 |
| T1 to T3 | 0.009 | 0.0173 |
| T2 to T3 | 0.0245 | 0.0173 |

**Table 8** Tukey test results for root width (cm).

## Discussion

### *The effect of synthetic hormones*

T1 containing IBA at 2,000 ppm has the best statistical results for average root length (3.36 cm) and root area (4.90 $cm^2$). Moreover, it scored 4.4 out of 5 in the rooting index, indicating that it produced a good root system and 95 per cent rooting percentage.

These results reinforce the idea of applying synthetic auxins in cutting propagation as they accelerate root formation and increase the percentage and uniformity of rooting as well as the number and quality of roots per cutting (Ruter, 2008).

### *The effect of natural hormones: lentils*

T2 has the poorest results of all treatments. It scored less than the control treatment for all the measured variables: rooting index, rooting percentage, length, width and area.

These results suggest that there is a possible presence of growth inhibitors in the extract. However, no inhibitors were found in the liquid chromatography results. Liquid chromatography results (Appendix A) show the presence of amino acids, organic acids and glucose. One possibility is that the concentration of the solution may have been toxic: Thimman (1977) states that crude plant extracts are frequently toxic. Additionally, the presence of excess micronutrients can inhibit root growth as roots are the first organ to accumulate the nutrient (Hopkins & Norman, 2009).

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### *The effect of natural hormones: willow*

T3 performed better than the control in length, width and root area. It had the best score of all treatments in the rooting index (4.55 out of 5) and its rooting percentage was 95 per cent, the same as the synthetic treatment.

These results suggest that there was a rooting effect that is not too different from the synthetic effects. Furthermore, willow scored the highest in the rooting index and in width, while synthetic scored highest for root area and length. Both had the same rooting percentage (95 per cent). These results reinforce the idea that products made with willow extract can naturally provide the same success rates as synthetic rooting hormone products (Hopper, 2016).

*Salix* species contain root-promoting hormones such as IBA and SA (Deep Green Permaculture, 2009). Liquid chromatography results (Appendix A) show that the extract contains SA, organic acids and amino acids. SA was only recently recognised as a plant growth regulator and it triggers defence reactions in the whole plant against pathogens. In addition, research shows that when SA is applied with IAA, it stimulates adventitious rooting (Cheng *et al.*, 2008).

Liquid chromatography results (Appendix A) did not find evidence of auxin at high concentrations but rooting was significant. Auxin might be present at lower concentrations, however, and the Orbitrap mass spectrometer cannot intercept it (David Watson, pers. comm.). Instead, there is a presence of L-Tryptophan, an important amino acid from which IAA is derived (Hopkins & Norman, 2009; The International Plant Growth Substances Association, 2017). Moreover, the failure of specific cells to produce L-Tryptophan lowers their capacity to produce IAA, which impairs root growth (Overvoorde *et al.*, 2010).

Lecrerc & Chong (1983) stated that the rooting process is influenced by a balance of growth regulators or other rooting substances, rather than by a single substance. The chemical substances found in willow extract could have worked in synergy with the auxin (IAA). In fact, Kawase (1964) found a strong synergy of willow extract with IAA, which supports this idea of the influence of synergists or rooting cofactors.

## Conclusions

Based on the present study and wider literature, willow could be a reliable alternative to synthetic treatments in both agriculture and horticulture.

In an economic context, the use of synthetics such as IBA is not authorised in organic agriculture (Montero-Calasanz *et al.*, 2013). Furthermore, the new regulations for organic agriculture in the EU market will enter into force on 1 January 2021. These rules forbid the use of synthetics, including IBA, and they will also apply to non-EU farmers who export their organic products to the EU market (Europa.eu, 2018). In this context, it is necessary to find an alternative to synthetics, and this could be a niche for natural extracts. Further research decoding the identity of rooting substances or cofactors present in these natural extracts should enhance their value and utility in future. Also, in the context of the global biodiversity loss (CBD.int, 2011), it is of vital importance to develop effective propagation protocols, as outlined in the principles of the Target 8 Project (PlantNetwork, 2009), that could help for both *in situ* and *ex situ* conservation programmes for this species.

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## Appendices

### *APPENDIX A – Results of MS liquid chromatography of lentils and willow*

The following table shows the top 50 metabolites by intensity in willow in negative ion mode.

| Row m/z | Row retention time | Name |
|---|---|---|
| 289.0718 | 14.3 | (+)-Catechin |
| 96.96026 | 6.9 | Sulfate |
| 123.0452 | 5.3 | o-Methoxyphenol |
| 127.0403 | 6.0 | (4E)-2-Oxohexenoic acid |
| 341.1093 | 5.6 | Sucrose |
| 373.1138 | 5.4 | Secologanate |
| 191.0201 | 7.1 | Citrate |
| 353.0889 | 7.4 | Chlorogenate |

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| | | |
|---|---|---|
| 121.0294 | 6.2 | Benzoate |
| 165.0404 | 6.4 | L-Arabinonate |
| 245.0819 | 14.3 | Columbianetin |
| 173.1045 | 9.1 | L-Arginine |
| 259.0224 | 5.4 | D-Glucose 6-phosphate |
| 191.0564 | 6.0 | Quinate |
| 135.0297 | 6.3 | [FA trihydroxy(4:0)] 2,3,4-trihydroxy-butanoic acid |
| 89.02425 | 5.6 | (R)-Lactate |
| 331.0681 | 6.8 | 1-O-Galloyl-beta-D-glucose |
| 117.0195 | 6.7 | Succinate |
| 113.0245 | 5.6 | 2-Hydroxy-2,4-pentadienoate |
| 195.0511 | 6.1 | D-Gluconic acid |
| 96.96947 | 6.5 | Orthophosphate |
| 101.0242 | 6.0 | 2-Oxobutanoate |
| 393.0621 | 9.4 | Prostalidin A |
| 191.0563 | 7.6 | Quinate |
| 161.0456 | 5.9 | 2-Dehydro-3-deoxy-L-rhamnonate |
| 137.0245 | 7.2 | 4-Hydroxybenzoate |
| 179.0352 | 7.5 | 3-(4-Hydroxyphenyl)pyruvate |
| 174.9562 | 6.0 | Methylenediphosphonate |
| 125.0244 | 7.1 | Phloroglucinol |
| 111.0088 | 7.0 | 2-Furoate |
| 137.0242 | 6.0 | 4-Hydroxybenzoate |
| 129.0193 | 6.7 | Mesaconate |
| 109.0295 | 6.2 | p-Benzenediol |
| 221.0819 | 14.3 | [FA (12:4/2:0)] 2E,4E,8E,10E-Dodecatetraenedioic acid |
| 131.0712 | 6.0 | 6-Hydroxyhexanoic acid |
| 267.0723 | 5.6 | Inosine |
| 87.00844 | 5.8 | Pyruvate |
| 143.0348 | 6.1 | 2,3-Dimethylmaleate |
| 168.0434 | 7.0 | Phosphodimethylethanolamine |
| 146.0458 | 6.8 | L-Glutamate |
| 329.2337 | 5.5 | [FA trihydroxy(18:0)] 9S,12S,13S-trihydroxy-10E-octadecenoic acid |
| 447.0942 | 7.6 | Carthamone |

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| | | |
|---|---|---|
| 131.0827 | 9.1 | L-Ornithine |
| 137.0244 | 14.5 | 4-Hydroxybenzoate |
| 313.0571 | 7.1 | 1-Salicylateglucuronide |
| 463.0888 | 7.7 | Quercetin 3-O-glucoside |
| 209.0303 | 7.0 | D-Glucarate |
| 254.9825 | 6.0 | Ascorbate 2-sulfate |
| 167.0213 | 6.5 | Urate |
| 179.0561 | 6.0 | D-Glucose |
| *190.0506 | 5.9 | 5-Hydroxyindoleacetate |

*low intensity but of potential interest

The following table shows the top 50 metabolites by intensity in lentils in negative mode

| Row m/z | Row retention time | Name |
|---|---|---|
| 329.2337 | 5.5 | [FA trihydroxy(18:0)] 9S,12S,13S-trihydroxy-10E-octadecenoic acid |
| 327.2182 | 5.5 | [FA trihydroxy] 9S,11R,15S-trihydroxy-2,3-dinor-13E-prostaenoic acid-cyclo[8S,12R] |
| 345.2283 | 5.3 | [FA hydroxy(18:0)] 9-hydroperoxy-12,13-dihydroxy-10-octadecenoic acid |
| 333.0591 | 5.0 | sn-glycero-3-Phospho-1-inositol |
| 323.0924 | 5.8 | [Fv Dimethoxy(9:1)] 5,6-Dimethoxy-[2″,3″:7,8]furanoflavanone |
| 267.067 | 5.8 | Formononetin |
| 596.1123 | 5.8 | 4-Sinapoyloxybutylglucosinolate |
| 357.0971 | 5.8 | [Fv Methyl,trimethoxy,hydrox] 3,4-Methylenedioxy-2′,4′,6′-trimethoxy-beta-hydroxychalcone |
| 253.088 | 5.7 | [Fv Hydroxy,Methox] 4′-Hydroxy-2′-Methoxychalcone |
| 337.0714 | 6.0 | [PK] 7-O-Methylsterigmatocystin |
| 259.0224 | 5.4 | D-Glucose 6-phosphate |
| 257.0788 | 9.6 | (1-Ribosylimidazole)-4-acetate |
| 245.0429 | 5.0 | Glycerophosphoglycerol |
| 401.1226 | 5.6 | [Fv Hydroxy] 4,2′-Dihydroxychalcone 4-glucoside |
| 325.2025 | 5.4 | [FA trihydroxy(2:0)] 9S,11R,15S-trihydroxy-2,3-dinor-5Z,13E-prostadienoic acid-cyclo[8S,12R] |
| 347.2438 | 5.3 | [FA hydroxy(4:0/18:0)] 9,10,12,13-tetrahydroxy-octadecanoic acid |
| 147.0461 | 6.1 | trans-Cinnamate |
| 211.1339 | 5.4 | [FA oxo(12:0)] 12-oxo-10E-dodecenoic acid |

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| | | |
|---|---|---|
| 341.1023 | 5.8 | [Fv Methyl,trimethox] 3,4-Methylenedioxy-2′,4′,6′-trimethoxychalcone |
| 241.0121 | 5.2 | D-myo-Inositol 1,2-cyclic phosphate |
| 313.1079 | 5.6 | [Fv Hydroxy,methox] 2′-Hydroxy-3,4,5-methoxychalcone |
| 283.0978 | 5.6 | [Fv] Isoliquiritigenin 4,4′-dimethyl ether |
| 171.0063 | 5.3 | sn-Glycerol 3-phosphate |
| 161.0614 | 6.0 | cis-1,2-Dihydronaphthalene-1,2-diol |
| 393.21 | 9.0 | [ST (2:0/2:0)] (7E)-(3S,6RS)-6,19-epithio-23,24-dinor-9,10-seco-5(10),7-choladiene-3,22-diol S,S-dioxide |
| 209.118 | 5.4 | (+)-7-Isojasmonic acid |
| 313.2386 | 5.6 | [FA hydroxy(18:0)] 9,10-dihydroxy-12Z-octadecenoic acid |
| 253.0513 | 5.9 | [PK] Chrysophanol |
| 267.1381 | 10.4 | 3-Hydroxyestra-1,3,5(10),6-tetraen-17-one |
| 188.0317 | 6.0 | Quisqualic acid |
| 251.0722 | 5.7 | 4′-O-Methylisoflavone |
| 211.0771 | 9.7 | (+)-cis-3,4-Dihydrophenanthrene-3,4-diol |
| 279.1031 | 5.6 | [FA hydroxy(18:0/4:0)] 6-hydroxy-7E,9E-Octadecadiene-11,13,15,17-tetraynoic acid |
| 307.0612 | 5.7 | [Fv Methyl(9:1)] 3′,4′-Methylenedioxy-[2″,3″:7,8]furanoflavanone |
| 323.1862 | 5.4 | [FA oxo,hydroxy(18:3)] 12-oxo-14,18-dihydroxy-9Z,13E,15Z-octadecatrienoic acid |
| 263.0721 | 5.6 | [Fv Hydroxy] 2′-Hydroxyfurano[2″,3″:4′,3′]chalcone |
| 386.0575 | 5.9 | 4-Pentenylglucosinolate |
| 387.1667 | 5.4 | Tuberonic acid glucoside |
| 258.0383 | 5.3 | D-Glucosamine 6-phosphate |
| 148.0394 | 8.9 | 5,6-Dihydroxyindole |
| 421.0752 | 5.2 | Alpha,alpha′-trehalose 6-phosphate |
| 373.1138 | 5.4 | Secologanate |
| 293.0979 | 5.6 | N-Glycosyl-L-asparagine |
| 221.1181 | 5.4 | (+/-)-6-Hydroxy-3-oxo-alpha-ionone |
| 211.0771 | 11.1 | (+)-cis-3,4-Dihydrophenanthrene-3,4-diol |
| 301.202 | 5.4 | [FA hydroxy(16:0/2:0)] 9-hydroxy-hexadecan-1,16-dioic acid |
| 190.0506 | 5.9 | 5-Hydroxyindoleacetate |
| 477.1742 | 9.3 | glcNAc-1,6-anhMurNAc |
| 192.0171 | 8.3 | Creatinine phosphate |
| 135.0297 | 6.3 | [FA trihydroxy(4:0)] 2,3,4-trihydroxy-butanoic acid |

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The following table shows the top 50 metabolites by intensity in willow in positive ion mode.

| Row m/z | Row retention time | Name |
|---|---|---|
| 118.0863 | 6.7 | L-Valine |
| 104.0707 | 7.7 | 4-Aminobutanoate |
| 175.1188 | 9.1 | L-Arginine |
| 132.1019 | 6.7 | L-Leucine |
| 90.05509 | 6.8 | L-Alanine |
| 146.0811 | 6.8 | [FA oxo,amino(6:0)] 3-oxo-5S-amino-hexanoic acid |
| 132.1019 | 7.6 | L-Leucine |
| 116.0707 | 6.5 | L-Proline |
| 90.055 | 2.5 | L-Alanine |
| 148.0604 | 6.4 | L-Glutamate |
| 138.0549 | 6.9 | Anthranilate |
| 166.0863 | 6.8 | L-Phenylalanine |
| 120.0656 | 6.5 | L-Threonine |
| 118.0862 | 2.5 | L-Valine |
| 76.03948 | 6.9 | Glycine |
| 90.05502 | 11.8 | L-Alanine |
| 133.0607 | 6.6 | L-Asparagine |
| 144.1019 | 6.9 | Stachydrine |
| 114.0551 | 7.3 | (S)-1-Pyrroline-5-carboxylate |
| 98.98432 | 6.4 | Orthophosphate |
| 116.0706 | 3.5 | L-Proline |
| 76.03942 | 10.1 | Glycine |
| 156.0767 | 8.5 | L-Histidine |
| 184.0733 | 6.8 | Choline phosphate |
| 182.0812 | 7.2 | L-Tyrosine |
| 205.0971 | 8.0 | L-Tryptophan |
| 268.1037 | 7.0 | Neuraminic acid |
| 106.05 | 6.6 | L-Serine |
| 147.1126 | 8.8 | L-Lysine |
| 114.0549 | 3.4 | (S)-1-Pyrroline-5-carboxylate |
| 111.0202 | 14.5 | Ethylphosphonate |

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| | | |
|---|---|---|
| 162.076 | 6.5 | L-2-Aminoadipate |
| 76.03942 | 12.0 | Glycine |
| 129.0659 | 7.2 | 5,6-Dihydrothymine |
| 76.0394 | 2.6 | Glycine |
| 115.0502 | 10.1 | 5,6-Dihydrouracil |
| 331.2839 | 13.6 | [GL (16:0)] 1-hexadecanoyl-rac-glycerol |
| 355.102 | 7.4 | Chlorogenate |
| 124.0393 | 6.5 | Nicotinate |
| 104.1069 | 8.2 | Choline |
| 164.0737 | 9.0 | S-Methyl-L-methionine |
| 154.0861 | 8.0 | Dopamine |
| 355.0962 | 5.7 | (S)-N-[3-(3,4-Methylenedioxyphenyl)-2-(mercaptomethyl)-1-oxoprolyl]glycine |
| 148.0755 | 5.5 | 3-Methyloxindole |
| 127.039 | 7.2 | Phloroglucinol |
| 152.9947 | 4.5 | 3-(Hydrohydroxyphosphoryl)pyruvate |
| 144.1017 | 12.1 | Stachydrine |
| 116.0706 | 8.3 | L-Proline |
| 106.0499 | 11.8 | L-Serine |
| 128.0705 | 10.1 | 2,3,4,5-Tetrahydropyridine-2-carboxylate |

The following table shows the top 50 metabolites by intensity in lentils in positive ion mode.

| Row m/z | Row retention time | Name |
|---|---|---|
| 132.1019 | 6.7 | L-Leucine |
| 191.1137 | 9.3 | N-(omega)-Hydroxyarginine |
| 118.0863 | 6.7 | L-Valine |
| 104.0707 | 7.7 | 4-Aminobutanoate |
| 133.0607 | 6.6 | L-Asparagine |
| 166.0863 | 6.8 | L-Phenylalanine |
| 104.1069 | 8.2 | Choline |
| 116.0707 | 6.5 | L-Proline |
| 90.05509 | 6.8 | L-Alanine |
| 205.1293 | 9.0 | (+)-gamma-Hydroxy-L-homoarginine |

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| | | |
|---|---|---|
| 116.0706 | 8.3 | L-Proline |
| 175.1077 | 6.4 | N-Acetylornithine |
| 138.0549 | 6.9 | Anthranilate |
| 120.0656 | 6.5 | L-Threonine |
| 147.0764 | 6.3 | L-Glutamine |
| 182.0812 | 7.2 | L-Tyrosine |
| 133.097 | 8.6 | L-Ornithine |
| 156.0767 | 8.5 | L-Histidine |
| 175.1188 | 9.1 | L-Arginine |
| 106.0499 | 8.4 | L-Serine |
| 106.05 | 6.6 | L-Serine |
| 247.1439 | 8.2 | Hypaphorine |
| 184.0733 | 6.8 | Choline phosphate |
| 149.0919 | 8.8 | N5-hydroxy-L-ornithine |
| 146.081 | 8.3 | [FA oxo,amino(6:0)] 3-oxo-5S-amino-hexanoic acid |
| 150.0583 | 6.6 | L-Methionine |
| 132.1019 | 7.6 | L-Leucine |
| 162.076 | 6.5 | L-2-Aminoadipate |
| 148.0604 | 6.4 | L-Glutamate |
| 114.055 | 10.0 | (S)-1-Pyrroline-5-carboxylate |
| 76.03948 | 6.9 | Glycine |
| 164.0737 | 9.0 | S-Methyl-L-methionine |
| 146.0811 | 6.8 | [FA oxo,amino(6:0)] 3-oxo-5S-amino-hexanoic acid |
| 295.2264 | 9.6 | [FA oxo(5:1/5:0/8:0)] (1S,2S)-3-oxo-2-(2′Z-pentenyl)-cyclopentaneoctanoic acid |
| 113.0346 | 6.2 | Uracil |
| 176.1032 | 6.7 | L-Citrulline |
| 103.123 | 13.5 | Cadaverine |
| 232.118 | 5.9 | Suberylglycine |
| 203.1501 | 8.8 | NG,NG-Dimethyl-L-arginine |
| 161.1282 | 8.7 | N6-Methyl-L-lysine |
| 189.1596 | 9.7 | N6,N6,N6-Trimethyl-L-lysine |
| 295.2266 | 5.4 | [FA oxo(5:1/5:0/8:0)] (1S,2S)-3-oxo-2-(2′Z-pentenyl)-cyclopentaneoctanoic acid |
| 174.0871 | 9.2 | 5-Guanidino-2-oxopentanoate |

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| | | |
|---|---|---|
| 144.1019 | 6.9 | Stachydrine |
| 115.0502 | 10.1 | 5,6-Dihydrouracil |
| 129.0658 | 9.6 | 5,6-Dihydrothymine |
| 199.1439 | 7.5 | N-Pentenylglutamine |
| 139.0582 | 6.9 | 4-Methylthiotoluene |
| 124.0393 | 6.5 | Nicotinate |
| 128.0705 | 10.1 | 2,3,4,5-Tetrahydropyridine-2-carboxylate |

## *APPENDIX B – Mass spectrometer methodology*

Liquid chromatographic separation was carried out on an Accela HPLC system interfaced to an Exactive Orbitrap mass spectrometer (ThermoFisher™ Scientific, Bremen, Germany) using a ZICpHILIC column with a mobile phase consisting of 20 mM ammonium carbonate in HPLC-grade water (solvent A) and acetonitrile (solvent B), at a flow rate of 0.3 ml/min. The elution gradient was an A:B ratio of 20:80 at 0 min., 80:20 at 30 min., 92:8 at 35 min. and finally 20:80 at 45 min. The nitrogen sheath and auxiliary gas flow rates were maintained at 50 and 17 arbitrary units. The electrospray ionisation (ESI) interface was operated in both positive and negative modes. The spray voltage was 4.5 kV for positive mode and 4.0 kV for negative mode, while the ion transfer capillary temperature was 275°C. Full scan data were obtained in the mass-to-charge range of 75 to 1200 amu for both ionisation modes.

The MS system was fully calibrated prior to running according to manufacturer's guidelines. The resulting data were acquired using the XCalibur 2.1.0 software package (ThermoFisher™ Scientific, Bremen, Germany).

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