DISSERTATION

PHYTOHORMONE EFFECT ON PTEROCEPHALUS DEPRESSUS STOCK PLANT PRODUCTIVITY AND PHYTOHORMONE ACCUMULATION AND MOVEMENT

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ABSTRACT

PHYTOHORMONE EFFECT ON PTEROCEPHALUS DEPRESSUS STOCK PLANT PRODUCTIVITY AND PHYTOHORMONE ACCUMULATION AND MOVEMENT

Moroccan pincushion (*Pterocephalus depressus*) is a drought tolerant perennial that is being used in landscapes throughout arid areas of the western United States. Commercial producers have had difficulty in producing enough plants to meet demand for moroccan pincushion due to production and propagation stock plant problems. Producers of comparable ornamental perennials have increased their use of gibberellic acid 3 (GA_3) in stock plant production. The use of GA_3 has increased the yield of vegetative cuttings from perennial stock plants. The plant hormone GA_3 is involved in many physiological processes, including plant growth and development. In current literature, few reports are available on the interaction between exogenous GA_3 and other plant hormones and their effect on successful propagation of vegetative cuttings. However, the research clearly demonstrated that several different hormone interactions with GA_3 could beneficially affect the cutting's rooting physiological process. First, this study describes two experiments researching optimization of stock plant production. Moroccan pincushion stock plants received foliar applications of GA_3, benzyladenine, ethephon, or indole-3-butyric acid (IBA) plant growth regulators (PGR). Plant growth regulators were applied singularly and in combination with GA_3 to determine efficacy on stock plant production. A propagation study was conducted simultaneously to determine effects of these different PGR treatments on the rooting of moroccan pincushion cuttings. The stock plant study showed GA_3 + benzyladenine increased cutting production over other treatments. Fresh weight of moroccan

pincushion did not differ among treatments. While dry weight showed no differences in experiment 1, but in experiment 2 differences were observed. The GA_3 + IBA treatment had the greatest overall growth. Treatments that included GA_3 were all greater in average growth index [(height + width + width)/3] and differed from those without GA_3 being applied. The propagation experiments indicated rooting percentages did not differ among treatments. However, growers look for 100% rooting and GA_3 + IBA was the only treatment with 100% rooting percentage for both experiments indicating potential benefits. The second part of this paper was to determine the movement and accumulation of GA_3 and IBA in treated moroccan pincushion (*Pterocephalus depressus*). Plants were treated with GA_3 alone and in combination with benzyladenine, ethephon, or IBA by either a foliar or drench application method. The amount of GA_3 and IBA found in basal and apical sections of moroccan pincushion was analyzed using liquid chromatography/ mass spectrometry (LC/MS). Results shown that drench applications effected the movement of GA_3 when GA_3 was combined with IBA or benzyladenine. The movement of IBA was affected by drench applications the greatest when GA_3 + IBA were applied. Both GA_3 and IBA were found in the greatest abundance when plants were treated with GA_3 + IBA in apical areas of moroccan pincushion. Nutrients in the cuttings were also analyzed. Only potassium had a significant difference for the amount found when treated with GA_3 as a drench application. Other nutrients detailed in this study were not affected by different PGR treatments. This study highlights the beneficial effect of GA_3 on production of vegetative cuttings without adverse effects on successful rooting of the cutting.

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DEDICATION

Casey,

You made me dream when I simply saw. You made me execute when I simply thought. You made me listen when I simply heard. You made me live every moment when I simply existed. Love you.

To Phyllis, I miss you every day and I wish my mom were here to see me finish this long trek.

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CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Ornamental herbaceous perennials are a major crop in the horticultural industry. The need for more landscape plants throughout the United States due to increased land development has amplified the demand for a larger market presence of herbaceous perennials. The United States Department of Agriculture's 2014 Census of Horticultural Specialties states that sales of potted herbaceous perennial plants were $945 million. This market showed an increase of 12% from the previous census taken in 2009, or a 2.25% annual increase in sales (USDA Census, 2014).

Ornamental herbaceous perennials are a major contributor to the landscape industry. They have become popular with consumers and industry professionals because of their advantageous low-input cultural characteristics. Low-input cultural characteristics include their drought, cold, and salt tolerances. These characteristics are often prevalent in the state of Colorado. The USDA reported that Colorado had $16.1 million in revenue for potted herbaceous perennial revenue for 2014 (USDA Colorado Census, 2014). These characteristics have resulted in increased overall production in the state of Colorado. However, with this increase in production more propagation problems have arisen for many growers.

Herbaceous perennials are highly desirable for many homeowners throughout the arid, intermountain region. One program administered through Colorado State University, the Denver Botanic Garden, and the Colorado Green Industry is the Plant Select® brand. Plant Select® is the country's leading brand of plants designed to thrive in High Plains and Intermountain Regions, offering plants that provide more beauty with less work. Gardeners of all levels

utilizing these plants can achieve smart, stunning, and successful gardens using less resources and results in a positive environmental impact (Plant Select, 2017).

Greenhouse and nursery operations propagating these Plant Select® plants indicated that there are production problems with Moroccan Pincushion (*Pterocephalus depressus* Archibald). The main problems indicated were slow growth rates of this 'petite' perennial and/or low propagation rooting percentages. These problems could have solutions by using plant growth regulators (PGR). Previous research performed at Colorado State University with PGR on four Plant Select® herbaceous perennials have shown gibberellic acid increased cutting production. In this study gibberellic acid alone and in combination with other PGR was applied either foliarly or by drench on stock plants of *Pterocephalus depressus.*

1.2 Background Information on *Pterocephalus depressus* Moroccan Pincushion

Pterocephalus depressus is an herbaceous perennial in the family Caprifoliaceae (honeysuckle family). It thrives in well-drained soil and full sun with low mat-like evergreen leaves, short-stemmed pincushion like pink flowers that end in attractive silvery seed heads (Plant Select, 2017). A native of the Moroccan Atlas Mountains, this perennial is well suited in high elevations of the Rocky Mountains. This species is closely related to the genus *Scabiosa* (pincushion flower) and possesses similar flowers. Not much research is reported with *Pterocephalus depressus* propagation and growing. However, medicinally important *Pterocephalus* species have been researched for their possible healing values, especially in Asia (Akhgar and Safavinia, 2016).

The species *Pterocephalus hookeri* (C.B. Clarke) is a popular Tibetan herb that has been widely applied in many Tibetan prescriptions and has multiple traditional uses in the treatment of

illnesses such as cold, flu, rheumatoid arthritis, and enteritis in China (Gulcemal et al., 2010). The medicinal uses have led researchers to study specific compounds within the plant to isolate any beneficial compounds that could be extracted.

1.3 Vegetative Herbaceous Perennial Propagation

The selection and propagation of plants is one of the oldest works of mankind (Wells, 1971). The Royal Horticultural Society *Dictionary of Gardening* defines a cutting as "… any portion of a plant, root, stem, leaf, or bud which is separated from the plant and has been induced to form roots of its own." (Royal Horticultural Society, 1999). Asexual plant propagation is used throughout the green industry for mass production of genetically identical plant crops through vegetative cuttings. Reasons for vegetative over sexual propagation include the inability to produce viable or true to type seeds, perpetuate a certain form of the plant, modification of habit, adaptability to habitat, and to develop pest resistance (Mahlstede et al., 1966). Hartmann et al. (2002) states that commercial propagators have developed technologies that successfully manipulate environmental conditions to maximize rooting, but what has lagged is the knowledge of the biochemistry, the genetic, and molecular manipulation of rooting.

The determination of the best time to take cuttings is critical for successful propagation process (Wells, 1971). One needs to know the plant nomenclature to be propagated, its specific biology, and cultural needs. Herbaceous perennials are very diverse, and it is difficult to determine many specifics for the propagation of an individual plant. The type of cutting to be taken is also an important determination for a propagator. Different types of cuttings are determined by area on the plant where cuttings are taken, apical (tip) or lateral and the specific plant organ, leaf, stem, and root (Wells, 1971). In this study, *Pterocephalus depressus* required an apical stem cutting based on Plant Select® propagator comments.

Once the specific type of cutting is determined the propagator then must decide on the use of PGR. Most commercial propagators use some form of auxin, either indole butyric acid (IBA) or 1-naphthaleneacetic acid (NAA) (Fretz et al., 1979). After speaking with Plant Select® propagators, it was determined that for *Pterocephalus depressus* a rate of 500 $mg \cdot L^{-1}$ (ppm) IBA is enough to aid in consistently high rooting percentage.

Each greenhouse operation is unique in their overall propagation protocols, the soilless medium chosen for propagation is an area where propagators have many choices. Common propagation soilless medium is perlite, vermiculite, sand, sphagnum peat moss, and pine bark; or some combination of several of them (Raviv and Lieth, 2008). The preference of the propagator is usually determined through trialing different media in different combinations on various plant species to determine the optimal selection. Propagation media should be readily available and inexpensive. Also, the medium should possess certain characteristics; uniform, long-lasting, good drainage, disease, insect, and weed-free (Fretz et al., 1979).

The use of additional heat in the root zone can be beneficial during the propagation process. Bottom heat for a propagation bench has been shown to increase rooting rates (Wells, 1971). The exact conditions a propagator prefers are based on experience gained with their growing environment and production procedures. A standard bottom temperature of 22 °C is commonly used by propagators as a basis when new varieties are propagated (Markovic and Klett, 2020a). If propagation success is not achieved, then adjusting the temperature is a common first step in finding an improved protocol.

Humidification and intermittent mist are important factors in the vegetative propagation process. The use of greenhouse systems to keep humidity levels high has shown to be of vital

importance for commercial propagation facilities (Wells, 1971). A critical task for the propagation is to determine the correct amount of moisture to be added to the propagation environment. Fog, direct mist, or the use of plant cloth material are humidifying aspects in greenhouse space. Greenhouse propagation areas can monitor and adjust the level of humidity within growing areas very precisely (Hartmann et al., 2002). The ability of greenhouse environmental monitoring allows the propagator to consistently produce high rooting percentages for several taxa.

Once the specifics of the plant, process, and the propagation environment to be used are determined, the original source of the propagation material plays a critical role in the overall propagation success. Stock plants, also known as mother plants, and their proper maintenance are needed to produce healthy propagation material (Dirr, 2009). A motto used at some propagation facilities is, "Start clean, End clean." This illustrates the desire for clean, healthy stock material for successful propagation results. When the preferred size, quality, and quantity of stock plants to be used are known to growers, positive results will occur.

1.4 Herbaceous Perennial Stock Plant Management Research

Managing stock plant health is an important part of propagation, it has been studied for many herbaceous perennials propagated throughout the United States. The concept of treating stock plants to increase rooting potential before cutting collection is an old concept. This approach recognizes that post cutting-collection treatments and propagation environments are rarely optimal. Cuttings with high rooting potential suffer least from any subsequent deficiencies (Howard, 1994). Like in many areas of horticulture, starting with healthy, disease free plants are desired. Procuring healthy cuttings to be planted and grown into stock plants is a key practice that must not be underestimated by propagators. Grower cultural practices have an impact on

overall health of stock plants (Lamb et al., 1975). Introducing pests to the propagation area should be avoided. Proper nutrition and plant care of stock plants are also required cultural practices. Most growers start new stock plants from plugs then allow the plants to grow for 6 to 12 weeks before harvesting cuttings (Gibson et al., 2005).

Growers have different procedures for harvesting cuttings, but they can be grouped into two general categories, selective or hedging harvests. Selective harvesting is when the grower removes only the 'best' available cuttings from the stock plants. While hedging is done by taking all the available cuttings that meet a certain standard that was predetermined (Gibson et al., 2005). The grower harvest method is based on genus of plant and production schedule. What works for some growers for a specific genus, might not work for others, based on their production schedule and procedure. Some growers only need a small number of cuttings at one time of year, while others are constantly propagating.

It is highly desirable to keep stock plants in a juvenile or vegetative state of development. Wells states, "The state of development of the cutting and its condition on removal from the parent are of the highest importance." He also states that the success of propagating the cutting is highly dependable on the judgement of the propagator (Wells, 1971). Reproductive tissue on cuttings can inhibit root and vegetative development (Gibson et al., 2005).

Plant nutrition is a cultural area that can have an impact on overall productivity of herbaceous perennial stock plants. Stock plant fertilizer concentrations for herbaceous perennials is typically between 150 to 200 $mg \cdot L^{-1}$ (ppm) Nitrogen (Gibson et al., 2005). A constant feed injection unit maintains nutritional levels in stock plants. Proper nutrition of stock plants helps maintain healthy, vigorous plants which result in superior cutting material.

Cultural practices growers have used to maintain a state of juvenility include lighting and temperature in the greenhouse space. The length of photoperiod required for herbaceous perennials varies based on the type of plant be they a short day, long day, or day neutral plant. Specific stock plant knowledge can be used to determine if additional lighting is required. Also, temperature manipulation can be easily programmed in most modern greenhouses. The knowledge of how to keep stock plants in a juvenile state will aid in developing propagation protocols for herbaceous perennials. *Pterocephalus depressus* has many unknowns about the required ideal conditions for maintaining juvenility. Researching the native region of this plant helps to determine if it is a long or short-day plant, for which *Pterocephalus depressus* is a long day flowering perennial (Denver Botanic Garden, 2020).

1.5 Plant Growth Regulators

Many chemicals are found in plants have effects on functions and growth. The substances that influence the reactions and metabolism within plants are hormones, which are internally synthesized (Meyer et al.,1960). Plant hormones are involved in many plant growth and development processes, which allow plants to respond to introduced internal or external stimuli (Rademacher, 2015). Phytohormones, another term for plant hormones, are naturally occurring organic chemicals that are synthesized at a given site and usually translocated to the site of action in the plant. The five major phytohormones are auxin, cytokinin, gibberellin, abscisic acid, and ethylene. Plant growth regulators (PGR) are any synthetic and natural chemical that shows hormonal effects (Hartmann et al., 2002).

Plant growth regulators aid in propagation, to increase yield, to improve plant quality, to alter plant growth habit, or to aid in harvesting or postharvest storage (Preece et al., 1993). The application of PGR in commercial greenhouse operations is widely used, but there are some

areas of the industry where increased knowledge and research into effects of PGR would be beneficial. Most PGR are typically applied via foliar sprays with water as the carrier. This application method can be easily incorporated into most commercial systems (Rademacher, 2015). Plant growth regulators used in the study will be discussed in further detail.

1.5.1 Gibberellic Acid

Gibberellins also known as Gibberellic Acid (GA) promotes growth primarily through cell elongation which is uniform throughout the plant tissue. Plant growth is cell division and cell elongation. Gibberellins and auxins are two special growth-regulating chemicals that effect cell elongation (Salisbury and Kriedemann, 1969). The plant stem growth resulting from GA treatments is due to the increased elongation of cells as well as an increase in cell division. Gibberellic acid also influences plant metabolism in several ways: stimulating cell division by the enhancement of DNA and RNA synthesis, hydrolyze starch into sugar, which in turn provides energy and encourages uptake of water by cells, cell wall elasticity is another activity of GA (Moore, 1984). Gibberellic acid utilized for PGR have been isolated from species of the fungus *Gibberella fujikuroi* and were first found in Japan in 1926 by E. Kurosawa (Salisbury et al., 1969).

Gibberellins are diterpenoids, which means they contain four isoprene units. An isoprene unit is five carbon atoms bonded together to form a molecule shaped like a capital Y. Gibberellins all basically have the same four-ring molecular structure (Fig. 1.2); but they differ in the total number of carbons, some have 19 while others have 20 carbons. Gibberellic acids also can possess different side chains (Preece et al., 1993). GA is found in a wide range of plant parts including meristem, roots, stem, and the seed embryo. Gibberellins are transported

throughout the plant in the xylem and phloem and occur during numerous stages of growth. GA applied to one part of the plant can have effect on other plant parts (Leopold et al.1975).

Gibberellic acid production is not done synthetically, but through the process of fermentation of *Gibberella* fungi. During the fermentation process GA are separated out and concentrated into different GA. Gibberellic acid number 3 (GA_3) is the most popular GA used by members in the green industry for cell elongation and the ability to break seed dormancy (Preece et al.1993). For this study, GA_3 was used to determine cell elongation effects on *Pterocephalus depressus*.

Gibberellic acids are involved in a variety of plant processes. Seed germination and dormancy are two areas that GA has been shown to effect plant growth and development. Barnes (2013) stated that GAs are found in high concentrations in immature seeds and can offset the need for seed stratification. Gibberellic acid terminates seed dormancy by changing the seed coat permeability and activating specific enzymes such as amylases, which are enzymes that catalyze the hydrolysis of starch into sugars. Flower bud formation has also been observed with the use GA. Boyle, et al, (1994) cited an inverse relationship between vegetative growth and flowering due to a highly significant negative correlation between the numbers of flower buds per plant and new apical phylloclade per plant in Easter cactus.

The product used in this study, GibbPro produced by Valent U.S.A. Corporation (Walnut Creek, CA, www.valentpro.com), contains 4% GA_3 which has shown to retard the aging process in plants (Nelson, 2003). Keeping stock plants juvenile longer was one goal of this study. Increased stem elongation and juvenility are areas necessary for stock plant management to meet the economic demands of the overall production operation.

Gibberellic acid 4 is less persistent than GA_3, which can be better for propagation, where long lasting effects may be unwanted (Rademacher, 2015). Gibberellic acid 3 longevity may not affect the successful rooting of cuttings after 2-4 weeks in the plant. Gibberellic acids may inhibit adventitious root development and affect lateral root branching (Preece and Read, 1993). The goal of this study is to produce more cuttings, have cuttings root at a higher success rate and determine what effect GA_3 may have.

1.5.2 Benzyladenine

In the early 1900's it was known that certain substances caused increased cell division (cytokinesis). In 1913 G. Haberlandt, an Austrian scientist discovered soluble substances that were present in the phloem that could cause cell division in potato parenchyma cells (Salisbury et al., 1969). In 1954, Carlos Miller found that aged or autoclaved DNA from herring sperm would stimulate cell division of tobacco in tissue culture, this substance was called kinetin (Salisbury et al., 1969). The common name cytokinin is used for any chemical substance that stimulate cell division, or cytokinesis.

Cytokinins have been found to be involved in nearly all aspects of plant growth and development (Leopold et al., 1975). Other cytokinins have been discovered and many of them are isolated from plant tissues, beginning with zeatin discovered in corn (*Zea mays*). Zeatin is a modified version of adenine (Moore, 1984). Natural and synthetic cytokinins include: zeatin, zeatin riboside, kinetin, isopentenyladenine (2iP), and benzyladenine (BA or BAP).

Cytokinins are usually made up of adenine with a five-carbon isoprene as a side chain. The isoprene unit comes from the mevalonate pathway which is also where GA come from. Therefore, to a certain extent, gibberellins and cytokinins share a portion of the same

biosynthetic pathway in the cell (Preece et al., 1993). The biosynthesis of cytokinins of the purine type occurs via the substitution of the side chain onto the common plant constituent adenine (Leopold et al., 1975). Cytokinins are known for cell enlargement, not cell elongation like with auxins and gibberellins. They promote cell growth in all directions (Preece et al., 1993). Cell division promotion may result in decreased apical dominance when cytokinin levels in the plant are elevated (Hartmann et al., 2002).

The ratio of cytokinin to auxin has been studied and has been found to have a major effect on plant growth development (Preece et al., 1993). Higher auxin to cytokinin ratios results in better rooting, while higher cytokinin to auxin rations result in better vegetative growth (Preece et al., 1993). The increase in cytokinins in the plant through additional applications can have detrimental effects on the rooting percentages of herbaceous perennials (Grossman, 2012). Increasing branching and providing more propagation material per stock plant is important; however, having quality cuttings that produce roots at a high percentage is also important.

N-6-Benzyladenine (6-BA), Figure 1.3, is a synthetic cytokinin and was used in this study to determine selected plant species response for lateral branching. Cytokinins are used in a variety of horticultural practices. In commercial greenhouse production, cytokinins are applied to increase branching and help decrease crop times by increasing the ability of the plant to grow in a container for a shorter period. In micropropagation (tissue culture), cytokinins are incorporated in the agar for increased branching of plantlets for division (Barnes, 2013). The wide use of cytokinins in micropropagation can be a possible indicator for whole plant application success.

1.5.3 Ethephon

Ethylene is a gaseous plant hormone that affects a wide range of plant growth and development processes (Simons, 1984). In its pure form ethylene is a gas and at normal temperatures dissipates into the atmosphere too quickly to be effective if it is applied to horticultural crops (Preece et al., 1993). A main plant response to ethylene is the enhancement of maturation. Depending on the plant growth stage several responses are capable of being induced when ethylene is applied including seed germination, root hair development, flowering, increased branching, growth regulation, fruit maturation, and leaf drop (Nelson, 2003). These desirable responses to ethylene have led to the need for a nongaseous, liquid form of ethylene.

Ethylene movement in plants is by diffusive processes, due to the relatively small size of the molecule. The small size and the solubility in water and other lipophilic systems allows for easy movement of ethylene throughout the plant tissues. The easy movement through cell membranes is due to solubility in lipophilic systems. Movement through air spaces suggests porosity of the tissue allows for movement similar to carbon dioxide movement in the plant (Leopold et al., 1975). The easy movement of ethylene in the plant is the reason that ethylene affects many different growth and development processes in the plant.

The biosynthetic pathway of ethylene was studied by Lieberman and Mapson (1964), they first proposed that the amino acid methionine is the precursor of ethylene. Adams and Yang (1979) worked to establish the exact sequence for the ethylene biosynthesis pathway in ripening apples. The pathway that occurs is Methionine to SAM (S-adenosylmethionine) to ACC (1 -aminocyclopropane-1 -carboxylic acid) to ethylene. Methionine is first converted to S-adenosylmethionine (SAM) through reaction with available ATP. The next step in the pathway is the conversion of SAM to ACC and MTA (methylthioadenosine). ACC synthase, which

catalyzes the conversion of SAM to ACC and MTA has a key role in the regulation ethylene biosynthesis (Adams and Yang, 1979). Through this process ethylene is made available to the plant cells.

The liquid form of ethylene, ethephon, is widely used as an alternative to the gaseous form which allows for better efficacy on plant crops. The chemical name for ethephon is 2-chloroethyl phosphonic acid and is written as CEPHA in some instances. The structure of ethephon is a phosphonic acid compound having a 2-chloroethyl substituent attached to the phosphate atom, as in Figure 1.3. The 2-chloroethyl phosphonic acid compound breaks down in the more pH basic environment in the cell cytoplasm to release ethylene.

Ethephon enters the plant and begins to breakdown into three molecules phosphate, chloride, and ethylene. These are released into the plant systems and effect plant growth and development (Preece et al., 1993). The production of ethylene in a plant has been observed to occur slightly before the ripening process of fruit (Salisbury et al., 1969). Ethephon has been used on food crops since the middle of the twentieth century. The release of ethylene has been used to promote the maturation and ripening of apples, bananas, tomatoes, and coffee. Ethylene aids in the loosening of certain fruit to increase production efficiency. Cherries and walnuts are two major food crops that are treated with ethephon prior to harvest to obtain uniformity (Preece et al., 1993). The fruit industry uses Florel, a commercially available PGR that contains 3.9% ethephon, to increase efficiency in harvests through the release of ethylene (Nelson, 2003).

Ethephon is widely used to promote axillary shoot development and not damage the apical meristem (Hayashi et al., 2001). Other pinching PGR have more of a damaging effect on the plant growth than ethephon, which makes it a preferred chemical for many herbaceous

horticultural crops. The main response examined in this study, the inhibition of flowering initiation and abortion of young flowers has been written about in extensive detail by Dole and Wilkins (2005). The increase of branching and decrease of flower development can result in herbaceous perennial stock plants with significantly more vegetative cutting material.

1.5.4 Auxin

Auxins were discovered trying to explain a correlation effect called phototropism. Charles Darwin (1897) found that the tip of a grass coleoptile is essential to the tropistic response of the whole coleoptile. The curvature was thought to be from a correlation carrier. Went (1928) found a substance that diffuses from coleoptile tips causing the tropism effect, auxins. Auxins generate many different responses from the plant such as: apical dominance, shoot elongation, organ differentiation, induction of cambial cell division, and root initiation (Buchanan et al., 2000). The responses are due to the auxin shaped gene expressions that regulate growth and development in intact plants as well as excised stem and root cuttings (Beyl and Trigiano, 2015). In this study, auxin is looked at for the ability to initiate root growth.

The auxin present in most plants is indole-3-acetic acid (IAA) (Leopold et al., 1975). IAA content in plant tissues is regulated by several processes. L-tryptophan synthesis, non-tryptophan precursors, and hydrolysis of IAA conjugates are what feeds the pool of IAA in plant tissues (Buchanan et al., 2000). In this study, the non-tryptophan precursor is most relevant because indole-3-butyric acid (IBA) is used as the applied PGR (figure 1.4) and IBA is synthesized from IAA (Buchanan et al., 2000).

The biosynthesis of IBA in *Zea mays* involves IAA as the direct precursor (Ludwig-Mueller, 2000). Conversion of IBA to IAA has been demonstrated in a variety of plants (Fawcett

et al., 1960) and involves b-oxidation of the four-carbon carboxyl side chain of IBA to the two-carbon side chain of IAA (Liu et al., 2012; Fawcett et al., 1960; Zolman et al., 2007). As a form of auxin storage, the carbon chain lengthened compound IBA, requires peroxisomal b-oxidation to IAA for auxin activity (Strader et al., 2010; Bartel et al., 2001; Woodward and Bartel, 2005). The exogenous IBA applied in this study was added for the purpose of root initiation of the cutting material. The extra IBA within the plant due to the exogenous application should increase the overall IAA concentration in the tissue and result in an increased number of initiated roots.

1.6 Herbaceous Perennial Response to Plant Growth Regulators Research

Research pertaining to the application of PGR on herbaceous perennial crops increased in the past twenty-five years. Commercial operations are interested in any product that may allow them to lower their input costs or decrease the growing time required for finishing herbaceous perennial crops. Research specifically involving GA, benzyladenine, ethephon, and auxin on herbaceous perennials has been conducted on many commercial taxa. Unfortunately, no research on *Pterocephalus* has been reported with PGR. Although, parallels can be drawn between similarly growing herbaceous perennials and *Pterocephalus*.

Bluebird Nursery in Clarkson, Nebraska has used GA_3 since the early 1990's and found that applying a product named GibbPro (Abbot Laboratories, Chem & Ag Products, North Chicago, IL) at a rate of 25 mL per 10 L on 4-inch pots of *Heuchera sanguinea* 'Snow Angel' which resulted in increased cutting numbers (Ackerman et al.1994). They noticed a two to three times rate increase in vegetative growth as well as increased axillary bud development. This study was done at the nursery, but did not involve a control group, so results are not statistically valid. However, the continued use of GA_3 on *Heuchera* 'Snow Angel' by the nursery and the

improved vegetative growth results are encouraging to do further research of GA on this herbaceous perennial.

Research performed on *Heuchera sanguinea, Epilobum canum* ssp. *garrettii, Salvia pachyphylla,* and *Osteospermum* x 'Avalanche' involving GA, benzyladenine, and ethephon have taken place at Colorado State University over the past four years. Results from these studies have been published and indicate a great potential for GA and benzyladenine in stock plant production (Markovic and Klett, 2020a, 2020b). Increased production of cutting material from all four taxa resulted from application of Fascination®, which is a commercial PGR that combines GA_{4+7} and benzyladenine (Markovic and Klett, 2020a).

Growth promotion by benzyladenine may result from increased concentrations of chlorophyll and other photosynthetic components (Song et al., 2013) and changes in leaf anatomy, this has been described in other species (Gandolfo *et al*., 2014). The exogenous application of benzyladenine can have similar effects. In addition, benzyladenine was shown to increase the cytokinin to auxin ratio in the plant and increase lateral branching by disrupting apical dominance (Cline, 1991). The use of benzyladenine on herbaceous perennials has proven to be beneficial in efficacy and improved branching. In the past two decades, benzyladenine has been researched thoroughly on many herbaceous perennials. In a study that involved herbaceous perennial liners with applications of 300, 600, 900, and 1200 $mg \cdot L^{-1}$ benzyladenine showed increased branching on *Echinacea* at rates as low as 300 m $mg \cdot L^{-1}$ (Latimer et al. 2011).

The application of benzyladenine on *Dianthus caryophyllus* was researched in Poland on stock plant production and resulted in more cuttings, except for the highest application rate of 800 $mg \cdot L^{-1}$ (Mynett, 1977). The application of benzyladenine on *Hylotelephium* 'Autumn Joy'

was shown to be very effective on treated liners resulting in four times as many lateral branches when compared to control (Latimer et al., 2013). Potential application schedules for stock plants to result longer-term production plants is an important factor. It has been shown that over time treated, and untreated liners eventually resulted in the same amount of lateral branching after only one treatment (Grossman et al., 2012). Possibly more favorable results can be achieved through an intensive application schedule at a shorter interval.

Latimer et al. (2015) found that *Heuchera* 'Silver Lode' had little response to an application of 600 $mg \cdot L^{-1}$ benzyladenine for plant height and width. The compactness of the *Heuchera* crown makes it difficult to accurately count lateral branching. An increase in branching was observed after destructive harvests. Benzyladenine applications increased in branching, which resulted in more propagation material on some herbaceous perennials. Martin and Singletary (1999) noticed an increase in lateral offshoots was accompanied by more uniform offshoot growth, which resulted in less production time and more uniform cuttings.

The PGR ethephon breaks down and releases ethylene, which influences internode elongation, increases branching, and aborts reproductive buds (Lopez et al., 2017). Some early research with ethylene was performed by Warner and Leopold in 1967. They determined that the Amchem Products compound 66-329 controlled the release of ethylene better than any other plant regulator used, which were mainly auxins (Warner et al., 1967). The wide array of plant activities that ethephon influences resulted in an increase of PGR research on herbaceous perennials. The commercially available product Florel has been researched for its efficacy on herbaceous perennials. Ethephon applications increased vegetative growth and controlled the timing of flowering. Ethephon treatments on herbaceous perennials resulted in increased number of cuttings while also reducing the size of the cutting (Brown et al., 2000).

Konjoian (1994) performed research with Florel and its effect on many greenhouse crops including both annuals and perennials. These studies were responsible for the increased desire to find greenhouse crops and production processes that could benefit from ethephon applications. Konjoian (1994) estimated an 80% reduction in labor with the application of ethephon by eliminating the need for hand removal of flowers and the promotion of vegetative growth. Also, Whipker (2015) found that using ethephon on vegetative annuals improved plant structure, prevented early flowering, and controlled excessive plant growth.

Roger C. Styer (2002) reported proper application rate, timing, and crop susceptibility for Florel. Florel can control of plant height and promotion of branching. He stated that Florel was cheaper than most other PGR and more cost effective than pinching or cutting by hand. Styer (2002) found utilizing Florel on stock plants to increase branching instead of hand pinching or in coordination with can result in increased production efficiency. A study at Texas A&M University found that out of 27 vegetative annuals only three displayed no response to 500 and 1000 $mg \cdot L^{-1}$ ethephon applications (Starman et al., 2004). Ethephon effect differently the growth and development of a wide range of herbaceous annual plants. Therefore, further research into ethephon should be conducted on new herbaceous perennials.

Environmental factors could influence the efficacy of ethephon in the plant. Air temperature and water alkalinity are two factors studied. It was determined that air temperature at the time of application should be below 26 °C and high alkalinity water should be buffered before tank mixing (Lopez, 2017). The application of ethephon is usually done through foliar spray, but recent research has suggested drenching can result in more uniform effects on the greenhouse crops (Aiken et al., 2015). Reduction in stem elongation as well as a flowering delay was observed on research performed on a broad range of annual floriculture crops, although

biomass accumulation was reduced (Miller et al., 2012). When a drench is performed the substrate pH can have an impact of the efficacy of ethephon on herbaceous perennials. *Verbena* and *Veronica* both had media pH responses to an ethephon drench performed a week after transplanted. As the substrate pH increased the ethephon drench showed less effect on the plant growth (Aiken et al., 2015). The ethephon in the substrate dissociates before the plant can take up the compound and have the desired effects within the plant.

Michigan State University has researched PGR on the production of herbaceous perennials. Dr. Erik Runkle maintains a website dedicated to PGR information on herbaceous perennials and annuals (http://www.flor.hrt.msu.edu/PGRs/). One study conducted at Michigan State University performed by Glady et al. (2007) showed the effects of ethephon on three herbaceous perennials. Growers inability to control plant growth and development through environmental signals has led to attempts to control these processes through chemical control. It was found that the effect of ethephon was species dependent. Weekly and biweekly treatments of 400, 600, and 800 $mg \cdot L^{-1}$ ethephon resulted in markedly different responses on *Veronica, Coreopsis,* and *Dianthus.* Other herbaceous crops have also shown the species-specific sensitivity to ethephon application. The timing and repetition of application also effected cutting quality and stock plant growth (Glady et al., 2007). Research on new herbaceous perennials is required before ethephon applications can be recommended for these new crops.

1.7 Hormone Interaction Research

The interaction between plant hormones has been studied and basic knowledge about how GA, cytokinin, ethylene, and auxin effect one another is available. Part of this study is to determine the interaction between GA_3 and benzyladenine, ethephon, and auxin. Research has been conducted on some of these interactions, GA_3 hormonal interactions with other hormones

has been reported with rice (*Oryza sativa*), *Arabidopsis thaliana*, and tomato (*Solanum lycopersicum*). However, no herbaceous perennial research has been done to date.

In *Arabidopsis,* the antagonistic relationship between benzyladenine and GA_3 has been studied and it has been observed to affect the growth of the plants through decreased signaling for both hormones (Greenboim-Weinberg et al., 2005). The negative effects stated in this experiment make the findings of the positive effects with Fascination® (Valent USA Corp., Fresno, CA) applied on stock plants of *Heuchera, Salvia*, and *Osteospermum* more interesting (Markovic and Klett 2020a, 2020b). The GA_{4+7} and benzyladenine hormone components of Fascination® would be in contrast with what was found when GA_3 and benzyladenine were applied to *Arabidopsis*. Also, research performed on tomato with the same interactions had similar findings to those of *Arabidopsis* (Fleishon et al., 2011). In tomato it has been found that GA inhibits cytokinin signaling in its response pathway. While cytokinin is also thought to affect the downstream branch(es)of the GA signaling pathway (Fleishon et al., 2011). The GA to cytokinin ratio is an important factor for this interaction, not the overall concentrations. This could be why the effects of Fascination® are beneficial to increased stock plant productivity, while other studies found antagonistic results for the hormone interaction.

The relationship between ethylene and GA_3 has been studied. Signaling between the two hormones during times of stress such as flooding in rice or stress induced floral initiation (Kuroha et al., 2018; Achard et al., 2007). In rice fields where flooding occurred, rice would grow taller to be above the water level. In this research, it was stated that ethylene concentrations increased and signaled the plant to increase GA concentrations. The greater GA concentrations caused the elongation of the internodes and the plant would grow taller above the water level. Therefore, a beneficial effect of the hormone interaction was observed (Kuroha et al., 2018).

Auxin and GA have been shown to promote many of the same plant responses such as internode elongation and apical dominance. The auxin and GA interaction has been studied and there is evidence that auxin promotes GA biosynthesis in pea (*Pisum sativum*) (Ross et al., 2000). Auxin like GA is mainly synthesized in the apical meristem and it was found that auxin from the apical meristem would transport down into the stem where it directly or indirectly helped maintain enzymes involved in GA biosynthesis (Ross et al., 2000). While auxin was included in this study mainly for its positive effect on root initiation, this advantageous interaction with GA could have an unforeseen benefit for stock plant production.

1.8 Study Objectives

The objectives of the stock plant study for *Pterocephalus depressus* were divided into an applicable component for growers and a basic component for understanding the role GA_3 plays in propagation through movement within the stock plant and effect on nutrient concentrations within cuttings. The applicable component was to determine if PGR treatment(s) resulted in more vegetative propagation material with high propagation qualities. We hope to develop stock plant protocols for growers to improve their propagation rates to be more productive and profitable. The propagation study objective was to determine whether the stock plant protocol resulted in any effects on the rooting percentages for the cuttings produced. The basic science component was to determine the concentration and movement of GA_3 within the stock plant to look at GA_3 as a singular application and in combination with benzyladenine, ethephon, and IBA. Also, compare the nutrient levels of phosphorus, potassium, and magnesium in the harvested cuttings for each treatment and determine the effect PGR treatments have on nutrient concentrations. These objectives provide the basis for expanded knowledge about how PGR

effect the biochemistry of the stock plant and specifically what happens when they are applied to *Pterocephalus depressus*.

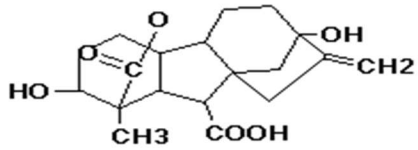

Figure 1.1 Molecular structure of Gibberellic Acid provided by www.planthormones.info.

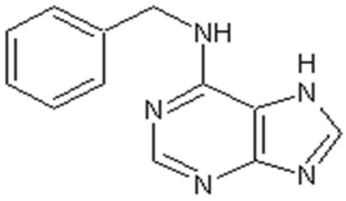

Figure 1.2 Molecular structure of N-6-Benzyladenine provided by www.sigmaaldrich.com.

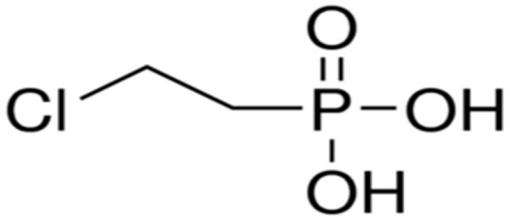

Figure 1.3 Molecular Structure of Ethephon provided by www.sigmaaldrich.com.

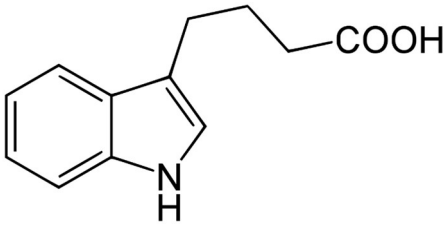

Figure 1.4 Molecular structure of indole-3-butyric provided by www.sigmaaldrich.com.

CHAPTER 2. MATERIALS & METHODS

2.1 Herbaceous Perennial Stock Plant PGR Study

This study was conducted at the Colorado State University Horticulture Center, which is located at 1707 Centre Avenue, Fort Collins, CO. The first experiment was performed starting in February 2019 with data collected through February 2020. The second experiment was performed starting in June 2019 with data collected through April 2020.

This research was designed to examine propagation and cutting production of an herbaceous perennial in the Plant Select® program: *Pterocephalus depressus* Moroccan Pincushion (Archibald). Plants of uniform size (72 plug tray) were purchased from a local greenhouse (Gulley Greenhouse, Fort Collins, CO). A total of 135 plants were selected, so that four replicates of three plants (twelve total) were placed in a randomized complete design and placed throughout the greenhouse bench for each of the ten treatments and control group (Fig 2.1).

The plants were transplanted from the 72-count plug into black 10 cm containers. All containers were first soaked in a disinfecting anti-fungal, anti-bacterial, and anti-algae solution of GreenShield and water for ten minutes prior to use to prevent contamination from previous use. The media used for this study was a sphagnum peat substrate composed of blonde peat moss, dolomitic limestone, and a wetting agent (Pindstrup, Ryomgaard, Denmark).

Groups of twelve plants were randomly selected for a specific PGR treatment. Four PGR were applied: ethephon [250 and 500 $mg \cdot L^{-1}$ (ppm)] (Verve, Nufarm Americas, Inc., Alsip, IL), benzyladenine (200 and 400 $mg \cdot L^{-1}$) (Configure; Fine Agrochemicals Limited, Worcester,

U.K.), Gibberellic Acid 3 (GA_3) (25 mg·L^{-1}) (GibbPro; Valent USA Corp., Fresno, CS), indole-2-butyric acid (200 mg·L^{-}1) (Hortus IBA Water Soluble Salts; Hortus USA Corp., Netherlands), and a control group was maintained. PGR were applied solo and in combination with GA_3. The foliar treatments were applied using a 3.79-liter hand pump sprayer starting two weeks before the first data collection and then two weeks before each collection throughout the duration of the two experiments. While the drench treatments were applied as a bottom drench where the 4-inch containers were placed in a flat with 1-gallon PGR solution for 1 hour. The overview of the experiment and number of plants is detailed in appendix 2.1.

The first experiment treatments were applied on July 1, 2019, August 8, 2019, September 20, 2019, and November 6, 2019. The second experiment treatments were applied on August 8, 2019, September 20, 2019, November 6, 2019, and January 24, 2020. The harvest of cuttings was performed approximately two weeks after the PGR treatment applications. These treatments were based on the recommendations on the product label and from interviews of nine Colorado greenhouse growers, who have previously or are currently growing these taxa.

The experiments were placed on a single rolling greenhouse bench with dimensions approximately 1.54 m by 12.19 m. The four groups of 3 plants for each treatment were randomly assigned a location on the greenhouse bench using random number generation in Microsoft Excel, making the layout as a complete randomized design. Groups of three were space approximately 30 cm apart. The plants were individually numbered 1 to 132 and data was collected separately for each plant.

The greenhouse used for this study was run by a control system. The greenhouse, number 118, was heated by a natural gas, forced air heater, and cooled passively by automatic ridge vents

and automatic pulled shade cloths, and actively by a pad and fan system. Daytime temperatures were maintained between 16.7 and 20 degrees Celsius during the day with a night-time range of between 12.8 and 16.7 degrees Celsius. No supplemental lighting was used during these experiments, this decision was made by the research team to suppress flowering of other genera in the greenhouse.

Stock plants were watered by hand when over 75% of the plants had visibly dry soil with a 14-4-14 fertilizer (GreenCare; Blackmore Co., Belleville, MI) at 200 parts per million (ppm) nitrogen every watering. Fertilizer was constantly injected (Dosatron® model D14MZ2 Clearwater, FL). Some pesticide treatments were applied during these experiments. Fungicides and insecticides were used in rotations to control fungal and insect pests. No pest populations were established on the stock plants during either experiment.

2.2 Cutting Protocols

Protocol for *Pterocephalus depressus* cutting harvest:

Superior cuttings will have a width at base (.25 to .5 cm stems) with no lateral shoots. Clean the cutting by removal of dead leaves and lateral buds.

Step by Step Protocol:

1. Start by taking most ideal cuttings, being careful not to remove more than 1/2 of total foliage.
2. If 1/2 of foliage is removed at this point, move to next plant; if not then continue by taking slightly fewer ideal cuttings until 1/2 of foliage has been removed or no acceptable cuttings remain.
3. Remove any dead foliage from the stock plant at this time.

4. Cut meristem off any shoots that are too large to take as a cutting (increase lateral growth for next round of cuttings).
5. Place all cuttings in labeled brown bag and record number of cuttings on bag.
6. Weigh (grams) cuttings for total fresh weight and record.
7. Place all bags in 70 °C drying oven for 48 hours. Record all total dry weights (grams).

2.3 Data Collection

Initial measurements of height and two widths were taken before the first application of PGR treatments for all 132 plants. Parameters measured were plant height, width, number of cuttings, total fresh weight of cuttings, and total dry weight of cuttings. Plants were measured in centimeters at their highest point from the base of the plant and at two perpendicular widths. Photographs were taken at each sampling date to help document the differences between the treatment groups, before cuttings were removed from the individual plants. Figure 2.3 illustrates the photographs taken and the visual differences between treatments.

The cuttings from each individual stock plant were counted, placed in a paper bag and weighed to determine the fresh weight, then placed in a drying oven at 70 degrees Celsius for 48 hours. After the cuttings were completely dried, the bags were weighed again to obtain the dry weights. After harvest, stock plants grew for 6-8 weeks before taking another set of cuttings.

In the first experiment, the first cutting harvest was taken July 17, 2019, the second cutting harvest on August 23, 2019, the third round on October 7, 2019, and the last round on November 22, 2019 for a total of four harvests. During the first round of cuttings the apical meristem was removed from each plant at that time to stimulate branching, this is a common

practice with all new *Pterocephalus* stock plants and a recommendation from Gulley Greenhouse.

In the second experiment, the first round of cuttings was taken August 23, 2019, the second round of cutting harvest on October 8, 2019, the third round on November 22, 2019, and the last round on February 11, 2020 for a total of four harvests. Again, during the first round of cuttings the apical meristem was removed from each plant at that time to stimulate branching.

Six weeks after the last cutting harvest for each experiment stock plants from each treatment had all the vegetative growth removed, dried, and weighed. This was done to simulate the average growth of the plant between harvest events. The root balls were removed from the pots and based on a determined rating scale of zero to five (zero being no roots and five being a fibrous root system), given a visual rating. A visual reference was photographed and displayed as root ratings were taken for the individual plants for consistency.

Prior to planting, samples of the Pindstrup media used in this study were submitted to Colorado State University's Soil, Water and Plant Testing Laboratory for analysis. Analysis included the percent lime, soluble salts, pH, Electric Conductivity (EC) and Cation Exchange Capacity (CEC) for the media. The analysis also determined the following: levels of nitrogen as ammonium, nitrate, and organic nitrogen, ratio of Ammonium: Nitrate, the Carbon: nitrogen ratio and total carbon in the media. Phosphorus content was measured as P and P_2O_5, while potassium content was measured as K and K_2O. Analysis included percent lime, soluble salts, pH, Electric Conductivity (EC) and CEC. Media test results are presented in appendix 2.2.

2.4 Rooting Study

Two stock plants from each treatment combination were randomly selected and grown under the same conditions for a rooting study. The only variables for rooting experiment were the stock plant treatments. Cuttings were harvested from each treatment combination every 6-8 weeks; August 23, 2019, October 7, 2019, and November 22, 2019 for the first experiment. Unfortunately, the second repetition (October 7, 2019) was a crop loss due to water drainage issues with the heating mats preventing water draining away from the plug flats. A replacement repetition was conducted on February 11, 2020 and this is the data used for analysis. For the second experiment, October 8, 2019, November 22, 2019, and February 11, 2020. Plug flats were placed on top of webbed flats to correct the drainage issue resulting from being directly on the heat mats where they were unable to drain.

Cuttings were taken at the same time of day, approximately 11:00 AM in the morning, and stuck in trays 72 cells filled with Jiffy® Preforma media and placed under mist with bottom heat at a temperature of 23.9 °C (Fig. 2.4). Rooting data was then collected after the second week up to four weeks. The rooting data collected was rooting percentage and the number of visual roots (counted to 30). Ten randomly selected cuttings from the two stock plants were chosen and then stuck in 72 cell trays. The plug trays were placed on web flats on top of heating mats that maintained a soil temperature of 23.9 °C. The mist times on the bench were adjusted weekly, for week one, ten seconds every 15 minutes, for the second week every 30 minutes and for the third and fourth weeks every 60 minutes. This was active for the total 24-hour period each day, there were no differences between mist intervals for day or night.

2.5 Nutrient Tissue Analysis

Inductively coupled plasma optical emission spectrometry (ICP-OES) was used to determine cutting nutrient content and its effect on cutting rooting success. This analysis enabled deeper understanding of the metabolic response of the cuttings resulting from PGR treatments. The nitric acid digestion procedure came from work done by Zarcinas et al. (1987). The protocol used for this analysis started with 100 mg of dried moroccan pincushion leaf tissue weighed and broken apart in a glass digestion tube. Then, in the chemical hood 1 mL of nitric acid (HNO3) was added to the dried material and a glass funnel was place on the tube to prevent evaporation. A blank sample was made by dispensing 1 mL of nitric acid into an empty digestion tube. The tubes were set in digestion blocks and spaced out to avoid cross contamination. The digestion program ran at a temperature of 60 °C for two hours and then 122 °C for six hours.

At the completion of the digestion program, labeled 15 mL tubes were used to transfer sample contents from the glass digestion tubes. Reverse osmosis treated water was added until the 10 mL level was reached in the test tube. The samples stored in racks on the lab bench until analyzed using ICP-OES. The process used during ICP-OES was performed as described by Winge et al. (1978) and is detailed in the appendix (A5.1). Results were generated into an excel file and calculated based on the exact initial dry weight which was recorded while weighing the plant material from the dried samples. Nutrients analyzed included: Ca,

The first set of samples were digested on October 21-23, 2019. These samples were analyzed with the ICP-OES machine November 19, 2019. The second set of samples were digested on March 5-6, 2020. These samples were analyzed with the ICP-OES machine March 16, 2020.

2.6 GA_3 Liquid Chromatography Mass Spectrometry

Liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) was used to quantify GA3 and IBA in different parts of the plant. The hormone concentrations in the apical and basal regions of the plant helped determine movement and accumulation of these hormones within the plant and allowed for interpretation of their effects on propagation success.

Two sample dates for each experiment were chosen for the first PGR application and last PGR application. The first experiment dates were at the beginning of the experiment on July 17, 2019 and the end of the experiment on November 22, 2019 and the second experiment dates were August 23, 2019 and February 11, 2020. On each sample date 110 samples were collected, five samples per treatment, two locations per plant, and eleven treatments. A total of 220 samples for experiments 1 and 2 were collected. Samples from apical and basal regions of *Pterocephalus depressus* were collected for each treatment involving GA_3 (five samples per treatment). The samples were collected and immediately (within 30 seconds) wrapped in aluminum foil and frozen in liquid nitrogen. The samples were then stored in a -80 °C freezer. After all the samples were collected the samples were then lyophilized using a freeze dryer (HarvestRight, North Salt Lake, UT) with pressure pump (Alcatel ADP81; Highvac Corp, Colorado Springs, CO). The lyophilized material was ground into a powder using a mortar and pestle under liquid nitrogen. The grinding process took less than a minute to complete and more liquid nitrogen added when necessary to keep the sample frozen. The samples were stored in 15 mL test tubes in a -80 °C freezer. This procedure is detailed in appendix 2.4.

2.6.1 Extraction

Five analytical replicates were prepared by adding 19-21-mg portion of ground leaf tissue to a 2-mL glass vial. Hormone were extracted by adding 500 µL of 80 % methanol in water

solution and vortexing at 4 °C for 3 hours. For a period of 16-hours samples were placed in a -20 °C freezer. After the 16-hour period, samples were centrifuged at 4 °C for 15 minutes at 3500×g. A 400 µL aliquot of the extraction was transferred to a fresh 2-mL glass vial, dried under nitrogen, re-suspended in 110 µL of methanol. Glass vials were then sonicated for 20 minutes and centrifuged at 4 °C for 20 minutes. A 100 µL aliquot of the extraction was transferred to a fresh 2-mL glass vial 100 µL of extraction was transferred to a new 2 mL vial insert, and then stored at -80 °C until LC-MS/MS analysis.

2.6.2 Ultra-High-Performance LC-MS/MS

Five microliters of plant extract were injected onto a LX50 UHPLC System, equipped with a LX50 Precision Sampling Module (20-µL sample loop, partial loop injection mode) (PerkinElmer, Waltham, MA, USA). An ACQUITY UPLC T3 column (1 × 100 mm, 1.8 µM; Waters Corporation) was used for chromatographic separation. Mobile phase A consisted of LC-MS grade water with 0.1% formic acid and mobile phase B was 100% acetonitrile. Elution gradient was initially 0.1% B for 1 min, which was increased to 55.0% B at 12 min and further increased to 97.0% B at 15 min, then decreased to 0.1% B at 15 min. The column was reequilibrated for 4.5 min for a total run time of 20 min. The flow rate was set to 200 µL/min and the column temperature was maintained at 45 °C. Samples were held at 4 °C in the autosampler. Detection was performed on a QSight™ 220 triple quadrupole mass spectrometer (MS) operated in selected reaction monitoring (SRM) mode. SRM transitions for each compound were optimized through analysis of authentic standards. The MS was operated with the ESI voltage 4500 V in positive mode and -3500 V in negative mode. Nebulizer gas flow was set at 350 arbitrary units and drying gas was set to 120 arbitrary units. The source temperature was 315 °C and hot-surface induced desolvation (HSID) temperature 200 °C.

2.7 Data Analysis

Data analysis was done using R version 4.0.2 with packages car, LSMeans, plyr, and ggplot2. A two-way ANOVA was performed separately for each response variable. Response variables include average number of cuttings per plant, average fresh weight per cutting, average dry weight per cutting, and the final dry weight of top growth, final root ratings. Terms included in the model were predictor variables matching to the plant growth regulator treatments (11 levels). Pairwise comparisons and least squares means were calculated using the lsmeans package for each response variable. Significant differences were noted using α=0.05 and 95% confidence intervals.

Response variables for the rooting study include average rooting percentage per treatment and average number of visible roots per plant per treatment. These were analyzed using a one-way ANOVA, pairwise comparisons, and least squares means were calculated using the lsmeans package for each response variable. Significant differences were noted using α=0.05 and 95% confidence intervals.

Response variables for nutrient tissue analysis data were the average amount of nutrients per treatment. These were analyzed using a One-Way ANOVA, pairwise comparisons, and least squares means were calculated using the lsmeans package for each response variable. Significant differences were noted using α=0.05 and 95% confidence intervals.

For UPLC-MS/MS analysis of GA_3 and IBA, Simplicity 3Q software (Version 4.1 SCN905, PerkinElmer, Waltham, MA) was used to detect and integrate peak areas and to calculate linear regression of analytical standards used for quantification. Each peak was normalized to an appropriate internal standard (IS). The corresponding linear regression equation

was used for quantification (ng/mL) for each analyte, which was then adjusted for precise weight of freeze-dried leaf tissue for each sample (ng/g). The limit of detection (LOD) was calculated as 3 times the standard deviation of the blank divided by the slope of the calibration curve. Likewise, the limit of quantitation (LOQ) was calculated as 10 times the standard deviation of the blank divided by the slope of the calibration curve. A Wilks-Shapiro test was run for both GA_3 and IBA, these tests indicated non-normal data for both parameters. A logarithmic transformation was performed to normalize the data, due to positive skewedness of the data.

Figure 2.1 Photograph of herbaceous perennial plant growth regulator study design layout on a single greenhouse bench, November 2019.

Figure 2.2 Photograph of *Pterocephalus depressus* cutting visual protocol (inches) provided by Gulley Greenhouse, Fort Collins, CO.

Figure 2.3 Photograph of treatments and control of *Pterocephalus depressus* stock plants February 2019, treatment from left to right: GA_3 + ethephon drench, GA_3 + benzyladenine drench, GA_3 + auxin drench, GA_3 drench, GA_3 + ethephon foliar, GA_3 + benzyladenine foliar, GA_3 + auxin foliar, GA_3 foliar, ethephon foliar, benzyladenine foliar, and control.

Figure 2.4 Propagation 72 cell trays with *Pterocephalus depressus* cuttings for all PGR treatments.

CHAPTER 3. RESULTS & DISCUSSION

3.1. Pterocephalus depressus Moroccan Pincushion

This chapter examines results from experiments 1 and 2 for *Pterocephalus depressus* in the format of a peer-reviewed journal manuscript. Results that are not included in this chapter are presented in the appendix.

3.2. Manuscript for *Pterocephalus depressus* Applicable Research

Plant Growth Regulator Impacts on Vegetative Cutting Production of Moroccan Pincushion

Sean J. Markovic and James E. Klett

3.2.1 Summary

Moroccan pincushion (*Pterocephalus depressus*) is a drought-tolerant perennial that is being used in landscapes throughout arid areas of the western United States. This paper describes two experiments researching vegetative cutting production from stock plants. Moroccan pincushion stock plants received foliar applications of gibberellic acid (GA_3), benzyladenine, ethephon, or auxin [indole-3-butyric acid (IBA)] plant growth regulators (PGR). Plant growth regulators were applied singularly and in combination with GA_3 to determine efficacy on stock plant growth. A propagation study was conducted simultaneously to determine effects of these different PGR treatments applied to stock plants on the rooting of moroccan pincushion cuttings. The stock plant study showed GA_3 + benzyladenine application increased cutting production over other PGR treatments. Fresh weight of moroccan pincushion cuttings did not differ among treatments. While cuttings did not differ in dry weight in experiment 1, but statistical differences

were observed in experiment 2. However, these differences in dry weight did not affect the quality of the cuttings. Cuttings from stock plants treated with GA_3 + IBA treatment had the highest numerical growth index [(height + width + width)/3]. Cuttings from stock plants treated with GA_3 alone or in combination with another PGR were all greater in average growth index and statistically differed from those without GA_3 being applied. PGR treatments did not affect rooting percentages of the cuttings with nontreated stock plant cuttings successfully rooting at an average rate of 95%. However, GA_3 + IBA was the only treatment where cuttings had 100% rooting for both experiments indicating potential rooting benefits.

Index words: Plant growth regulator, propagation, *Pterocephalus depressus*, vegetative cuttings.

Species used in this study: Moroccan Pincushion [*Pterocephalus depressus* (Archibald)].

Chemicals used in this study: gibberellic acid (GA_3), benzyladenine, ethephon, indole-3-butyric acid (IBA).

3.2.2 Significance to the Horticulture Industry

Moroccan pincushion (*Pterocephalus depressus*) is one of several perennials being evaluated as part of the Plant Select® landscape plants program at Colorado State University. As a drought-tolerant perennial ground cover, moroccan pincushion can provide a new option for drought affected areas in the western United States. The compact growth is ideal for use in smaller residential landscapes. The primary obstacle to further adoption of moroccan pincushion by producers thus far has been the lack of propagation success. Colorado State University researchers are developing production protocols for Plant Select® varieties, but these need to be proven successful before deployment to producers. Vegetative propagation is the most widely used method of propagation of moroccan pincushion. However, success with vegetative propagation has been variable. Stock plant quality has a large impact on the success of cuttings taken from moroccan pincushion. The use of gibberellic acid (GA_3), benzyladenine, ethephon, or

indole-3-butyric acid (IBA) may improve stock plant production and cutting fresh weight an indicator of cutting quality. We found that 0.025 $g \cdot L^{-1}$ (25 ppm) gibberellic acid (GA_3) in combination with 0.25 $g \cdot L^{-1}$ (250 ppm) benzyladenine was the best combination for increasing stock plant growth which produced a greater number of vegetative cuttings. Also, 0.25 $g \cdot L^{-1}$ (250 ppm) indole-3-butyric acid (IBA) can have positive effects on rooting success.

3.2.3 Introduction

Production of cuttings from herbaceous perennial stock plants can be challenging for producers. Stock plants in a juvenile state are desired for their high-quality propagation material. Reproductive tissue which occurs on cuttings will inhibit root and vegetative development during propagation (Gibson and Cerveny 2005). Producers often prune stock plants manually or use PGR to encourage vegetative plant growth and eliminate reproductive tissue (Preece and Read 1993). However, manually pruning perennials to encourage vegetative growth can be labor intensive (Banko and Stefani 1996) and thus increase production costs (Holland et al. 2007). Applying a PGR is generally less labor intensive than manual pruning, although there is a chance it can cause phytotoxicity in certain crops (Meijón et al. 2009). Research is required to determine PGR efficacy on new perennial species.

This research focused on utilizing gibberellic acid (GA), which increased cutting production in previous research on several herbaceous perennial stock plants (Markovic and Klett 2020). Gibberellic acid is isolated from a species of the fungus, *Gibberella fujikuroi* (Salisbury and Ross 1969), and has become a useful PGR in the ornamental horticulture industry. Gibberellic acid promotes growth primarily with uniform cell elongation throughout plant tissue

(Moore 1984). Therefore, application of GA on herbaceous perennials could result in more propagation material.

Cytokinins, specifically benzyladenine, are involved in nearly all aspects of plant growth and development (Leopold and Kriedemann 1975). Benzyladenine is known for cell enlargement, not cell elongation as with auxins and gibberellic acid, and it promotes cell growth in all directions (Preece and Read 1993). This results in decreased apical dominance if cytokinin levels in the plant are elevated (Hartmann et al. 2002). In tomato (*Solanum lycopersicum* L.), GA inhibits cytokinin signaling in its response pathway, while cytokinin is also thought to affect the downstream branch(es) of the GA signaling pathway (Fleishon et al. 2011). The ratio of GA to cytokinin is an important factor during interactions, not the overall concentrations. Positive interactions from applications of GA_{4+7} + benzyladenine on stock plants of coral bells (*Heuchera* sanguinea L.), mojave sage (*Salvia pachyphylla* Munz), and cape daisy (*Osteospermum species* L.) suggest a proper ratio of the PGR is important (Markovic and Klett 2020). Therefore, application of benzyladenine, which causes more lateral growth could produce more propagation material from stock plants.

Ethephon is an ethylene inducer which enters the plant and breaks down into three molecules: phosphate, chloride, and ethylene. These molecules are released into plant systems, effecting plant growth and reproductive development (Preece and Read 1993). These molecules promote auxiliary shoot development without damage to the apical meristem (Hayashi et al. 2001). The relationship between ethylene and GA has shown signaling between the two hormones during times of stress, such as flooding in rice (Oryza sativa. L.) or stress induced floral initiation (Kuroha et al. 2018, Achard et al. 2007). When applied ethylene signals the plant

to increase GA concentrations. Increasing the GA concentration increases internode elongation; therefore, a beneficial effect of this hormone interaction was observed (Kuroha et al. 2018). An increase in branching and decrease in flower development could lead to more vegetative growth on herbaceous perennials.

Auxins generate many different responses from the plant such as: apical dominance, shoot elongation, organ differentiation, induction of cambial cell division, and root initiation (Buchanan et al. 2000). Auxin and GA have been shown to promote many of the same plant responses such as internode elongation and apical dominance. The auxin and GA interaction has been studied and there is evidence that auxin promotes GA biosynthesis in pea (*Pisum sativum* L.) (Ross et al. 2000). Auxins, like GA are mainly synthesized in the apical meristem. Auxin from the apical meristem can transport down into the stem where it directly or indirectly maintains enzymes involved in GA biosynthesis (Ross et al. 2000). Auxin was included in this study mainly for its positive effect on root initiation. This advantageous interaction with GA could have an unforeseen benefit for stock plant cutting production.

Herbaceous perennial responses to PGRs can vary across cultural and environmental conditions (Cochran and Fulcher 2013). Coral bells and Orange Carpet hummingbird trumpet (*Epilobium canum* ssp. *garrettii* 'PWWG01S' L.) were found to have greater numbers of cuttings produced by stock plants when treated with GA_{4+7}, but the quality of the cuttings had a disparity with coals bells having high quality and hummingbird trumpet having low quality (Markovic and Klett 2020). Low quality cuttings had low fresh weights and low rooting percentages when propagated. The wide range of possible plant responses indicates the importance of to continue research on herbaceous perennials and their response to PGRs.

The herbaceous perennial utilized in these experiments was moroccan pincushion. During meetings with greenhouse and nursery growers propagating this perennial, growers identified two production problems with this plant: 1) lack of quantity of vegetative propagation material from stock plants and 2) too long of period between cutting collection. Based on prior research, it was concluded that using PGR could possibly resolve these problems.

The research objective was to evaluate moroccan pincushion stock plants response to GA_3 applied singularly or in combination with benzyladenine, ethephon, or auxin. The hypothesis of this study was that applications of GA_3 would increase vegetative cuttings numbers and quality from moroccan pincushion stock plants. A second hypothesis was that successful rooting of moroccan pincushion cuttings would not be affected by PGR applications to stock plants.

3.2.4 Materials and Methods

Stock plant study. Moroccan pincushion stock plants were treated with ten different plant growth regulator treatments to determine if they would produce more cuttings (Fig. 1). The stock plant experiment was repeated for moroccan pincushion, the first experiment was conducted during Summer and Fall 2019 and repeated in Fall and Winter 2019-20.

All experiments were conducted at the Colorado State University Horticulture Center greenhouse, Fort Collins, CO (lat. 40.577953º N, long. 105.080925º W; U.S. Department of Agriculture hardiness zone 5b). Plants of uniform size (98-plug tray) were purchased from a local greenhouse (Gulley Greenhouse, Fort Collins, CO). Over 135 rooted cuttings were potted into black, square 10 cm (4 inch) containers on 11 Feb. 2019 and 9 June 2019. The substrate used was composed of blonde peat moss, wood fiber, dolomitic limestone, and a wetting agent

(Pindstrup, Ryomgaard, Denmark). Four replicates of three (12 total plants) were then placed in a complete randomized design and placed in rows of 3 plants (same treatment) with an empty row between in 15-count carrier flats placed on a single greenhouse bench.

During establishment, plants were watered by hand with 14N-1.7P-11.6K water-soluble fertilizer (GreenCare; Blackmore Company, Belleville, MI) at a rate of 200 ppm. Fertilizer was applied during each watering using a handheld hose with breaker (400 PL, Dramm, Manitowoc, WI). Daytime temperatures were maintained with an aspirator (Model M4821, Wadsworth Control Systems, Arvada, CO) sensor between 18 and 23 C (65 and 73 F), while night-time temperatures were held between 16 and 22 C (61 and 73 F).

The four applied PGRs with application concentrations: auxin (indole-3-butyric acid): 250 ppm (Hortus USA Corp., New York, NY); ethephon: 400 ppm (Nufarm Americas, Inc., Alsip, IL); benzyladenine: 250 ppm (Fine Agrochemicals Limited, Worcester, U.K.); and GA_3: 25 ppm (gibberellic acid 3) (Valent USA Corp., Fresno, CS). Treatments in both experiments, were applied two weeks before each data collection over a 6-month period. In the first experiment, treatments were repeatedly applied on 12 June 2019, 8 Aug. 2019, 26 Sept. 2019, and 6 Nov. 2019. In the second experiment, treatments were repeatedly applied on 8 Aug. 2019, 26 Sept. 2019, 6 Nov. 2019, and 3 Feb. 2020. Treatments were applied to foliar run-off, including those in the control group, which were sprayed with plain water. All treatments were applied using a 3.78 L (1 gal) hand pump sprayer.

Initial measurements for height and width of each stock plant were taken after five weeks of growth after planting. Subsequently, stock plant height, stock plant width, number of cuttings, total cutting fresh weight (FW), and total cutting dry weight (DW) were measured during each

collection event. Stock plants were measured from the highest leaf to the substrate, across the plant, and then across at a 90-degree angle. These three values were added together and divided by three to determine the GI for each plant. Fresh weight was measured immediately after harvesting the cuttings from stock plants and this measurement was used to determine quality of the harvested cutting.

When cuttings were harvested, two-thirds of the total vegetative growth of the stock plant was left intact to ensure continual plant growth. This was suggested by commercial growers during discussions about production processes. Cuttings were harvested approximately two weeks after each PGR treatment application. Ideal cutting size and diameter are shown in Fig. 1. The ideal moroccan pincushion cutting has up to a 5 cm (2 inch) height and a stem with 0.3 cm (0.12 inch) diameter. Cuttings from each stock plant were counted, weighed to determine FW in grams, then placed in a drying oven at 70 C (158 F) for 48 h to determine DW in grams. One month after the fourth collection of cuttings, stock plants had all the shoot growth above the soil line removed, dried, and weighed. This provided average top growth of the stock plants for each treatment.

Propagation study. After each stock plant experiment, a propagation experiment was performed. Two stock plants for each treatment randomly selected from experiments 1 and 2 were grown under the same conditions. The only variable was the different PGR applied during the stock plant treatments. Three repetitions of the propagation experiments 1 and 2 were conducted with 10 cuttings being rooted in a completely randomized design in two 72-count propagation flats. Cuttings from moroccan pincushion were harvested every 4 weeks: 8 Aug. 2019, 26 Sept. 2019, 6 Nov. 2019 for the first experiment and 26 Sept. 2019, 6 Nov. 2019, and 3 Feb. 2020 for the second experiment. Cuttings were taken in the morning before 11:00 AM and

dipped for 30 s in 500 ppm indole-3-butyric acid/1-naphthylacetic acid (IBA/NAA) (Dip 'N Gro, Clackamas, OR) and propagated in trays of peat moss and binding agent (Preforma; Jiffy, Lorain, OH).

A mist timer (NOVA, 1626ET, Phytotronics, Earth City, MO) was used to control the amount of moisture administered on the cuttings which were under mist nozzles (03034211-b pcs 25 coolpro c 4x7 head +ad20, Netafim, Fresno, CA). Bottom heat was provided by heating mats (Redi Heat model RHD 2110, Phytotronics) at a temperature of 24 C (75 F). During the experiment, cuttings were misted for 10 s throughout the 24 h day at varying time intervals each week. Time intervals included: week 1, every 15 min; week 2, every 30 min; and weeks 3 and 4 every 60 min. Rooting data was collected weekly to 4 weeks after sticking. Plants were pulled out of the propagation tray cell to determine rooting percentage and visible roots were counted along the sides of the cell and returned to the tray. The data collected included rooting success percentage during weeks 2 to 4 on the mist bench. Also, the number of visible roots were determined by counting up to 30 individual roots in each of the cells.

Experimental analysis. Data analysis was performed using R version 3.3.1, statistical computing software (R Foundation for Statistical Computing, Vienna, Austria) with car, LSMeans, and ggplot packages. A one-way analysis of variance (ANOVA) was run separately for the response variables. Response variables included average number of cuttings per stock plant, average GI per stock plant, average FW per cutting, average DW per cutting, final average DW of total shoot growth per stock plant, average cutting rooting percentage per treatment, and average number of visible roots per treatment. The data were analyzed and averaged over the 4 collections and analyzed specific to each experiment (1 and 2). Included in the statistical models were predictor variables that matched the PGR treatments applied to the stock plants. Pairwise

comparisons and least squares means were calculated using the LSmeans package for each response variable. Tukey adjusted pairwise comparisons were considered and significant differences were noted using $\alpha=0.05$.

The propagation study had response variables with a combined sample size of 30 from three repetitions for each experiment. Data analyzed included successful rooting percentage and average number of visible roots per replicant. Data were analyzed using an initial arcsine transformation for the rooting percentages. Then, one-way ANOVA were run for rooting percentage and average number of visible roots for each experiment. Included in the statistical models were predictor variables matching PGR treatments. Pairwise comparisons and least squares means were calculated using the LSmeans package for each response variable. Tukey adjusted pairwise comparisons were considered and significant differences were noted using $\alpha=0.05$.

3.2.5 Results and Discussion

Number of cuttings harvested. The data from these experiments were averaged across all 4 harvest dates. There were no differences among the 4 harvest dates (data not shown). Foliar sprays containing GA_3 + benzyladenine (25 and 250 ppm) resulted in a greater number of cuttings harvested compared to all other treatments in experiment 1 and 2 (Fig. 2). The results were not statistically different when comparing all treatments together. However, statistical differences were observed in comparison to only the nontreated control, plants treated with GA_3 + benzyladenine (25 and 250 ppm) produced 2.59 (23.5%) more cuttings in experiment 1. While plants treated with GA_3 + benzyladenine (25 and 250 ppm) produced 2.49 (30.6%) more cuttings in experiment 2 compared to the control (Fig. 2).

More vegetative cuttings were harvested from stock plants treated with GA_3 + benzyladenine compared to all other treatments (Fig. 2). The additional lateral shoot growth of the stock plants combined with increased internodal elongation produced more available cuttings. Results for coral bells were comparable when treated with GA_{4+7} + benzyladenine (50 ppm) applications (Markovic and Klett 2020). Similarly, 'Bressingham Bronze' coral bells treated with benzyladenine (1000 ppm) resulted in more lateral shoots (Martin and Singletary 1999).

Benzyladenine (250 ppm) application resulted in a small increase in the number of cuttings harvested compared to the nontreated control due to increased lateral branching (Fig. 2). While ethephon (400 ppm) application resulted in similar growth to the nontreated control with no effect on the number of cuttings collected and were not statistically different (Fig. 2). No flowering was observed throughout the experiments on any treatment plants. Therefore, ethephon application did not affect reproductive tissue compared to other PGR treatments.

The application combination of GA_3 and IBA, ethephon, or benzyladenine increased the average number of cuttings collected. During the first experiment, stock plants treated with GA_3 + benzyladenine had a greater number of cuttings compared to the other treatments, while GA_3, GA_3 + IBA, and GA_3 + ethephon were all similar to each other. In the second experiment, GA_3 + IBA and GA_3 + benzyladenine treated stock plants produced greater numbers of cuttings and were statistically different than GA_3 + ethephon, which in turn differed from GA_3 alone (Fig. 2). The addition of another PGR to GA_3 showed positive interactions which increased the number of cuttings produced by stock plants, which follows the patterns in previous research performed on pea (Ross et al. 2000), rice (Kuroha et al. 2018), and coral bells (Markovic and Klett 2020).

Fresh and dry weight per cutting. Fresh weight has been a better indication of cutting quality than DW (Markovic and Klett 2020, Brown and Klett 2020). Fresh weight of moroccan pincushion in experiment 1 and 2 did not differ among treatments (Table 1). Dry weights showed no differences as with FW in the first experiment, but in experiment 2 differences were observed. The differences did not appear to have a significant effect on cutting quality.

Ethephon treated cuttings had the greatest DW and statistically differed from all other treatments (Table 1). Stock plants treated with the combination GA_3 + benzyladenine in experiment 2 had the least DW and statistically differed from all other treatments (Table 1). This did not affect the rooting percentage when compared to other PGR treatments. Low DW was observed in previous PGR research involving mojave sage, coral bells, and hummingbird trumpet when GA_{4+7} + benzyladenine were applied (Markovic and Klett 2020).

Growth index. There were no statistical differences in GI between treatments in the first experiment, but in experiment 2 statistical differences were observed (Table 1). The GA_3 + IBA treatment had the greatest overall growth increase, which differed from all other treatments. These results are comparable to those of Ackerman and Hamernik (1994) on coral bells. Treatments that included GA_3 were all greater in average GI and statistically differed from treatments without GA_3 applied. This could be expected due to GA_3 being involved with cell elongation (Moore, 1984). The GA_3 + ethephon treatment had the lowest average GI of all GA_3 treatments but was not statistically different (Table 1). This interaction was interesting because ethephon-treated stock plants had the least amount of growth during experiment 2. However, they had some of the greatest fresh weights, which was similar to mojave sage findings (Markovic and Klett 2020). Ethephon applications caused added growth through stem thickness of the plants, but not in height and width. These results contradict research performed on a broad

range of annual floriculture crops, where biomass accumulation was reduced with ethephon applications (Miller et al. 2012).

Propagation experiments. Successful rooting rates ranged from 63% with cuttings from ethephon treatment in experiment 1 to 100% with cuttings from GA_3 + IBA treatment in both experiments (Table 2). Although results indicated the rooting percentages did not significantly differ among treatments, 100% is what producers strive for in herbaceous perennials when propagating. The addition of IBA to stock plants suggests potential benefits for rooting success compared to other PGR treatments. Difficulty in propagating moroccan pincushion by growers was not observed during these experiments with nontreated stock plants producing cuttings with an overall 95% rate of successful rooting. The difference and quality of the propagation facility may be the underlying factor for successful propagation of moroccan pincushion.

The number of visible roots of cuttings did not statistically differ between treatments. While GA_3 + benzyladenine treated cuttings had the greatest average number of visible roots from experiment 1 (Table 2). Gibberellic acid 3 alone had the greatest average number of visible roots from experiment 2. Cuttings from both treatments did not repeat the same results during the other experiment. Therefore, no conclusions can be made on which treatment may benefit moroccan pincushion with a greater average number of roots.

This study was conducted to determine whether GA_3, benzyladenine, ethephon, or a combination of GA_3 and benzyladenine, ethephon, or IBA applications can improve the number of cuttings and successful rooting of cuttings taken from moroccan pincushion stock plants. Gibberellic acid 3 + benzyladenine application appears to have the greatest potential for improving propagation of moroccan pincushion. This can be attributed to the increase in plant

growth between cutting collections by GA_3, plus increased lateral growth from the addition of benzyladenine. The use of GA_3 + benzyladenine application increased number of cuttings harvested but produced minimal effect on the ability to improve rooting percentage or number of roots on cuttings. The application of IBA could be utilized to increase the rooting percentage of cuttings, however further research into applications of GA_3, benzyladenine, and IBA need to be studied to confirm potential benefits.

Table 3.1. Individual cutting fresh weight, individual cutting dry weight, and growth index of moroccan pincushion averaged over 4 harvest dates (Expt. 1 and Expt. 2) as influenced by seven plant growth regulator treatments: benzyladenine, ethephon, gibberellic acid 3 (GA_3), GA_3 + auxin, GA_3 + benzyladenine, GA_3 + ethephon, and control.

Treatment	Rate (ppm)	Fresh wt (g)	Dry wt (g)	Growth index (cm)
Expt. 1				
control	0	0.44	0.057	8.9
benzyladenine	250	0.43	0.056	8.8
ethephon	400	0.45	0.05	8.8
GA3	25	0.41	0.093	9.3
GA3 + auxin	25 + 250	0.42	0.044	9.5
GA3 + benzyladenine	25 + 250	0.42	0.049	9.5
GA3 + ethephon	25 + 400	0.41	0.05	9.3
P value		0.894	0.694	0.095
Expt. 2				
control	0	0.35	0.04ab	7.3ab
benzyladenine	250	0.33	0.04ab	7.6abc
ethephon	400	0.38	0.05b	7a
GA3	25	0.36	0.04ab	8.1cd
GA3 + auxin	25 + 250	0.34	0.04ab	8.6d

GA3 + benzyladenine	25 + 250	0.31	0.03a	8.3cd

[z]Treatments were applied foliarly, control received water only.

[y]1 ppm = 1 $mg \cdot L^{-1}$.

[x]Fresh and Dry Weights were taken as a total for each plant harvested and the average individual cutting weight was determined using total weight and dividing by the number of cuttings harvested from the single plant. 1g = 0.0353 oz.

[w]Growth Index (GI) determined from one height and two width measurements at the largest diameter cross-sections, equation GI = (Height + Width 1 + Width 2)/3; 1 cm = 0.3937 inch.

[v]Mean separation in columns with Tukey adjusted least squares means at $P \leq 0.05$ (lowercase letters).

Table 3.2. Influence on moroccan pincushion stock plants by seven plant growth regulator treatments: benzyladenine, ethephon, gibberellic acid 3 (GA_3), GA_3 + auxin, GA_3 + benzyladenine, GA_3 + ethephon, and control on cutting rooting percentage and number of visible roots. Data were collected after four weeks under mist and averaged over harvest date within Expt. 1 (Aug. to Nov. 2019) and Expt. 2 (Sept. 2019 to Feb. 2020).

Treatment	Rate (ppm)	Rooting Percentage	Average Number of Visible Roots
Expt. 1			
control	0	93	15.7
benzyladenine	250	83	16.8
ethephon	400	63	16.9
GA3	25	90	14.4
GA3 + auxin	25 + 250	100	14.6
GA3 + benzyladenine	25 + 250	83	18.6
GA3 + ethephon	25 + 400	90	15.2
P value			0.829
Expt. 2			
control	0	97	11.7
benzyladenine	250	100	10.3
ethephon	400	83	11.2
GA3	25	97	13.4

GA3 + auxin	25 + 250	100	13.3
GA3 + benzyladenine	25 + 250	93	12.9
GA3 + ethephon	25 + 400	100	13.2
P value			0.889

[z]Treatments were applied foliarly, control received water only.

[y]1 ppm = 1 $mg \cdot L^{-1}$.

Fig. 3.1. Visual guide for moroccan pincushion (*Pterocephalus* depressus) cutting protocol provided by Gulley Greenhouse, Fort Collins, CO. Cuttings show the ideal size and preparation for harvest. Measurements in inches (1 inch = 2.54 cm).

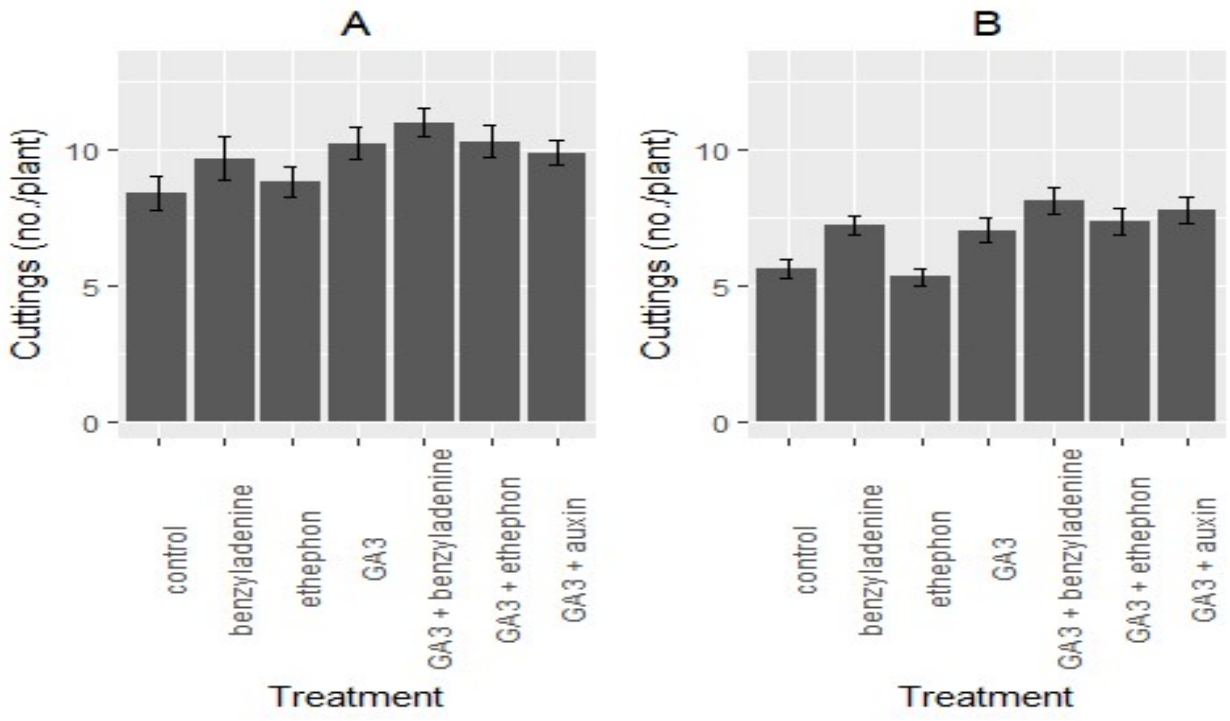

Fig. 3.2. Mean number of cuttings harvested per plant from moroccan pincushion stock plants averaged over four harvest dates for Expt. 1 (A) and Expt. 2 (B) as influenced by seven plant growth regulator treatments: benzyladenine, ethephon, gibberellic acid 3 (GA_3), GA_3 + benzyladenine, GA_3 + ethephon, GA_3 + auxin, and control.[z]

[z]Plant growth regulators were foliarly applied, with clear water on control plants until run-off occurred on leaves.

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CHAPTER 4. RESULTS & DISCUSSION

4.1. *Pterocephalus depressus* Moroccan Pincushion Phytohormone UPLC-MS/MS Experiment

This chapter examines results from UPLC-MS/MS experiment for *Pterocephalus depressus* in the format of a peer-reviewed journal manuscript. Results that are not included in this chapter are presented in the appendix.

4.2. Manuscript for *Pterocephalus depressus* Phytohormone LC-MS Experiment

Movement and Accumulation of Gibberellic Acid 3 in Response to Foliar and Drench Treatments in Moroccan Pincushion

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4.2.1 Summary

The plant hormone gibberellic acid 3 (GA_3) is involved in many physiological processes, including plant growth and development. Commercial producers of ornamental perennials have increased their use of GA_3 in stock plant production. The use of GA_3 has increased the yield of vegetative cuttings from perennial stock plants. In literature few reports are available on the interaction between exogenous GA_3 and other plant hormones and their effect on successful

propagation of vegetative cuttings. However, the published works clearly demonstrated that several hormone interactions with GA_3 could beneficially affect the physiological process of root development on cuttings. The aim of this research was to determine the movement and accumulation of GA_3 and Indole-3-butyric Acid (IBA) in treated moroccan pincushion (*Pterocephalus depressus*). Plants were treated with GA_3 alone and in combination with benzyladenine, ethephon, or IBA by either a foliar or drench application method. The amount of GA_3 and IBA found in basal and apical sections of moroccan pincushion was analyzed. Results shown that drench applications effected the movement of GA_3 when GA_3 was combined with IBA or benzyladenine. The movement of IBA was affected by drench applications the greatest when GA_3 + IBA was applied. Both GA_3 and IBA were found in the greatest abundance when plants were treated with GA_3 + IBA in apical areas of moroccan pincushion. This study highlights the beneficial effect of GA_3 on production of vegetative cuttings without adverse effects on successful rooting of the cutting.

4.2.2 Introduction

GA_3 is a plant hormone that regulates several aspects of plant growth and development. GA_3 promotes growth primarily through cell elongation which is uniform throughout the plant tissue (Moore, 1984). Plant growth is cell division, which involves the promotion of cell elongation. GA_3 is a growth-regulating chemical that effects cell elongation (Salisbury and Ross 1969). This research continued the focus on GA_3, which was previously researched on several herbaceous perennial stock plants resulting in increased cutting production (Markovic and Klett 2020a). GA is isolated from a species of the fungus, *Gibberella fujikuroi* (Salisbury and Ross 1969), and has become a useful plant growth regulator (PGR) in the ornamental horticulture industry.

We wanted to conduct further research into movement of GA_3 in perennials and how it is affected by other plant hormones. It has been reported that hormones act through a web of interacting responses rather than through isolated linear pathways (Kuppusamy et al. 2008). This signal integration architecture may be one mechanism for increasing the specificity of outcomes in different cellular contexts. We altered the balance between GA_3 and other hormones by exogenous applications to either the plant foliage or the root system. We then collected samples from apical and basal regions from the plants after the first application and fourth application. The purpose of these collection locations and times were to show where GA_3 accumulates within the plant. The combination of other hormones with GA_3 resulted in beneficial hormone interaction effects for increased GA_3 levels within plant tissue. Two different application methods provided a useful perspective on GA_3 movement throughout the plant.

Cytokinins, specifically benzyladenine, are involved in nearly all aspects of plant growth and development (Leopold and Kriedemann 1975). Benzyladenine is known for cell enlargement, not cell elongation as with auxins and gibberellic acid, which promotes cell growth in all directions (Preece and Read 1993). In tomato (*Solanum lycopersicum*), GA inhibits cytokinin signaling in its response pathway. While cytokinin is also thought to affect the downstream branch(es) of the GA signaling pathway (Fleishon et al. 2011). The ratio of GA to cytokinin is an important factor during interactions, not the overall concentrations. Positive interactions from applications of gibberellic acid 4+7 (GA_{4+7}) + benzyladenine on stock plants of coral bells, mojave sage (*Salvia pachyphylla*), and cape daisy (*Osteospermum species*) suggest a proper ratio of the PGR is important (Markovic and Klett 2020b).

Ethephon is an ethylene inducer that enters the plant and breaks down into three molecules: phosphate, chloride, and ethylene. These molecules are released into plant systems, effecting plant growth and reproductive development (Preece and Read 1993). The production of the gas ethylene may bring about changes within the same tissue, or within the same cell, where it is synthesized (Davies 2010). These molecules promote auxiliary shoot development without damage to the apical meristem (Hayashi et al. 2001). The relationship between ethylene and GA has shown signaling between the two hormones during times of stress such as flooding in rice or stress induced floral initiation (Kuroha et al. 2018, Achard et al. 2007). Ethylene concentrations increased and signaled the plant to increase GA concentrations. The increased GA concentrations elongated the internodes; therefore, a beneficial effect of this hormone interaction was observed (Kuroha et al. 2018).

Auxins produce growth responses away from its site of synthesis which fits the depiction of a transported chemical messenger. While auxins are usually transported within the plant and signal action at a distance that is not always the case. Auxins may have their intended action near the site of synthesis. However, auxin synthesis has been found to occur in a wide range of tissues throughout the plant (Davies 2010). Auxins generate many different responses from the plant such as: apical dominance, shoot elongation, organ differentiation, induction of cambial cell division, and root initiation (Buchanan et al. 2000). Auxin and GA have been shown to promote many of the same plant responses such as internode elongation and apical dominance. The auxin and GA interaction has been studied and there is evidence that auxin promotes GA biosynthesis in pea (*Pisum sativum*) (Ross et al. 2000). Auxin, like GA, is mainly synthesized in the apical meristem. It was found that auxin from the apical meristem would transport down into the stem

where it directly or indirectly helped maintain enzymes involved in GA biosynthesis (Ross et al. 2000).

The research objective was to evaluate and determine GA_3 and IBA levels in moroccan pincushion stock plants. Analyzing their response to GA_3 applied singularly or in combination with benzyladenine, ethephon, or IBA. The hypothesis of this study was that applications of GA_3 would increase vegetative cuttings numbers and quality for moroccan pincushion stock plants. A second hypothesis was a successful rooting of moroccan pincushion cuttings would not be affected by hormone applications.

4.2.3 Materials and Methods

Plant material and hormone treatment

Vegetative moroccan pincushion rooted cuttings were received from a local greenhouse (Gulley Greenhouse, Fort Collins, CO) and transplanted into 10 cm by 10 cm square pots containing peat substrate composed of blonde peat moss, wood fiber, dolomitic limestone, and a wetting agent (Pindstrup, Ryomgaard, Denmark). These were grown in a greenhouse with daytime temperatures monitored with an aspirator (Model M4821, Wadsworth Control Systems, Arvada, CO) sensor between 18 and 23 °C (65 and 73 °F), while night-time temperatures were held between 16 and 22 °C (61 and 73 °F). The effects of GA_3 (Valent USA Corp., Fresno, CS), auxin (IBA) (Hortus USA Corp., New York, NY), ethephon (Nufarm Americas, Inc., Alsip, IL), and benzyladenine (Fine Agrochemicals Limited, Worcester, U.K.) on hormone accumulation were assessed using plants of equal size.

Treatment applications were performed after roots were observed to strike the sides of the containers in most of the plants, about six weeks after transplant. A 3.78 L (1 gal) hand pump sprayer was used to apply GA3 (25 $mg \cdot L^{-1}$), IBA (250 $mg \cdot L^{-1}$), ethephon (400 $mg \cdot L^{-1}$), or

benzyladenine (250 mg·L^{-1}) on foliage of the whole plant. Trays with no drainage holes were filled with 3.78 L (1 gal) of each treatment and plant containers were placed in trays with 3.78 L treatment solutions for one hour to provide drench application. Treatments were applied four times with six-week intervals in-between each application. Five replicants of each treatment were carried out.

Apical and basal samples of at least one gram in weight were collected two weeks after the first and fourth hormone treatment applications on 22 Aug. 2019 and 20 Feb. 2020. All samples were rapidly frozen in liquid nitrogen and stored at -80 °C. Samples were freeze dried over a period of 36 hours in a freeze dryer (Harvest Right, North Salt Lake, UT), then ground to a fine powder with mortar and pestle in liquid nitrogen. Ground samples were stored at -80 °C for further phytohormone analysis.

Phytohormone extraction from moroccan pincushion tissues

Five analytical replicates were prepared by adding 19-21-mg portion of ground leaf tissue to a 2-mL glass vial. Hormones were extracted by adding 500 µL of 80 % methanol in water solution and 62.5 ng/mL internal standard solution, then vortexing at 4 °C for 3 hours. For a period of 16-hours samples were placed in a -20 °C freezer. After the 16-hour period, samples were centrifuged at 4 °C for 15 minutes at 3500×g. A 400 µL aliquot of the extraction was transferred to a fresh 2-mL glass vial, dried under nitrogen, re-suspended in 110 µL of methanol. Glass vials were then sonicated for 20 minutes and centrifuged at 4 °C for 20 minutes. A 100 µL aliquot of the extraction was transferred to a fresh 2-mL glass vial insert, and then stored at -80 °C until UPLC-MS/MS analysis.

Ultra-high-performance LC-MS

Five microliters of plant extract were injected onto a LX50 UHPLC System, equipped with a LX50 Precision Sampling Module (20-µL sample loop, partial loop injection mode) (PerkinElmer, Waltham, MA, USA). An ACQUITY UPLC T3 column (1 × 100 mm, 1.8 µM; Waters Corporation) was used for chromatographic separation. Mobile phase A consisted of LC-MS grade water with 0.1% formic acid and mobile phase B was 100% acetonitrile. Elution gradient was initially 0.1% B for 1 min, which was increased to 55.0% B at 12 min and further increased to 97.0% B at 15 min, then decreased to 0.1% B at 15 min. The column was reequilibrated for 4.5 min for a total run time of 20 min. The flow rate was set to 200 µL/min and the column temperature was maintained at 45 °C. Samples were held at 4 °C in the autosampler. Detection was performed on a QSight™ 220 triple quadrupole mass spectrometer (MS) operated in selected reaction monitoring (SRM) mode. SRM transitions for each compound were optimized through analysis of authentic standards. SRM transitions for GA_3 were 344.9 to 239.1 for a collision energy of 22 for the quantifier and 344.9 to 200.9 for a collision energy of 36 for the qualifier. SRM transitions for IBA were 204.1 to 117 for a collision energy of -46 for the quantifier and 204.1 to 130.1 for a collision energy of -38 for the qualifier. The MS was operated with the ESI voltage 4500 V in positive mode for IBA and -3500 V in negative mode for GA_3. Nebulizer gas flow was set at 350 arbitrary units and drying gas was set to 120 arbitrary units. The source temperature was 315 °C and hot-surface induced desolvation (HSID) temperature 200 °C.

Analysis of propagation of treated vegetative cuttings

Propagation success was determined using rooting percentage and number of visible roots as variables. Vegetative cuttings were collected and propagated using the method reported

by Markovic and Klett (2020b). The experiment was repeated three times and included ten replicates for each treatment.

Statistical analysis

For UPLC-MS/MS analysis of GA_3 and IBA, Simplicity 3Q software (Version 4.1 SCN905, PerkinElmer, Waltham, MA) was used to detect and integrate peak areas and to calculate linear regression of analytical standards used for quantification. Each peak was normalized to an appropriate internal standard which was added during the extraction process. The corresponding linear regression equation was used for quantification (ng/mL) for each analyte, which was then adjusted for precise weight of freeze-dried leaf tissue for each sample (ng/g). The limit of detection (LOD) was calculated as 3 times the standard deviation of the blank divided by the slope of the calibration curve. The LOD for GA_3 and IBA were 0.35 and 0.58, respectively. Likewise, the limit of quantitation (LOQ) was calculated as 10 times the standard deviation of the blank divided by the slope of the calibration curve. The LOQ for GA_3 and IBA were 1.06 and 1.76, respectively.

Data analysis was performed using R version 4.0.2, statistical computing software (R Foundation for Statistical Computing, Vienna, Austria) with car, LSMeans, plyr, and ggplot2 packages. A Wilks-Shapiro test was run for both GA_3 and IBA, these tests indicated non-normal data for both parameters. A logarithmic transformation was performed to normalize the data, due to positive skewedness of the data. Two-way analysis of variance (ANOVA) was performed separately for both response variables, GA_3 and IBA. Two-way ANOVA was performed for each hormone treatment and area of plant sampled or application method.

Propagation rooting percentages were analyzed using an initial arcsine transformation. Then, two-way ANOVA were performed for rooting percentage and average number of visible roots by treatment and application or location. Pairwise comparisons and least squares means were calculated using the LSmeans package for each response variable. Tukey adjusted pairwise comparisons were considered and significant differences were noted using α=0.05.

4.2.4 Results and Discussion

Effect of exogenous GA_3, benzyladenine, ethephon, and IBA on expression of GA_3 and IBA levels

The exogenous application of GA_3 combined with the three other hormones resulted in increased GA_3 levels when compared to control (Table 4.1). The addition of another PGR to GA_3 showed positive interactions, which follows the patterns in previous research performed on pea (Ross et al. 2000) and rice (Kuroha et al. 2018). Exogenous IBA combined with GA_3 resulted in the greatest levels of GA_3 found when compared to all other treatments (Table 4.1). The GA_3 plus IBA treatment was 24 times greater than control levels of GA_3. The positive interaction is what Ross et al. (2000) also reported with IBA, it transports down the stem where it directly or indirectly helped maintain enzymes involved in GA biosynthesis (Ross et al. 2000).

Benzyladenine combined with GA_3 also had increased GA_3 levels that differed from other treatments except when compared to the GA_3 and IBA treatment. It has been reported that cytokinin affects the downstream branch(es) of the GA signaling pathway (Fleishon et al., 2011). With additional GA_3 applied with the benzyladenine the ratio of the hormones allowed GA_3 to not be inhibited. We suspect application of benzyladenine without GA_3 would confirm what Fleishon et al. (2011) reported. Ethephon did not combine with GA_3 to produce levels of GA_3 that differed from GA_3 applied alone (Table 4.1). The relationship between GA_3 and ethylene

hormones has been linked to stress events such as flooding in rice (Kuroha et al. 2018). Plants in this study did not experience stress events that would have allowed for increased ethylene and GA_3 interaction. The beneficial relationship between GA_3 and IBA was most pronounced of all treatments for GA_3 levels within moroccan pincushion.

Treatments did not differ from control for increased IBA levels within the plant. Levels of IBA had the greatest increase when treated with GA_3 plus IBA (Table 4.1). These results were expected, as previous research demonstrated that exogenous GA has a positive impact on auxin signaling and transport (Li et al. 2015). When additional IBA was applied to the plant, IBA levels increased greater than 20 times when compared to control and four times greater when compared to the next greatest treatment, GA_3 plus benzyladenine (Table 4.1). The beneficial relationship between GA_3 and IBA was the most evident when compared to other treatments for increased IBA levels within moroccan pincushion.

Application method of exogenous GA_3, benzyladenine, ethephon, and IBA effects on expression of GA_3 and IBA levels

Exogenous foliar and drench application methods were analyzed to determine the effects of GA_3 and IBA accumulation. Overall, drench applications resulted in higher abundance of GA_3 when compared to foliar applications of treatments, while levels of IBA had greater amounts when foliar applications were utilized (Table 4.1). When moroccan pincushion plants were treated with GA_3 plus IBA by exogenous foliar applications GA_3 levels significantly differed when compared to all other treatments (Fig. 4.2). Auxin like IBA have been shown to promote GA synthesis in a range of systems, including shoots of tobacco (Wolbang and Ross, 2001), barley (Wolbang et al. 2004), and Arabidopsis (Frigerio et al. 2006). The treatment resulted in a 12% increase in GA_3 when compared to GA_3 plus benzyladenine which had the second greatest

level of GA_3. Also, exogenous foliar application of GA_3 plus IBA resulted in greatest IBA levels and differed from all other treatments except GA_3 plus benzyladenine (Fig. 4.3). The two greatest treatments had a 14% increase when compared to all other treatments.

Effect of exogenous GA_3, benzyladenine, ethephon, and IBA effects on expression of GA_3 and IBA levels in apical and basal regions

The apical and basal areas of the plant were sampled to determine movement of GA_3 and IBA within the plant when exogenous hormones are applied. The apical region of the plant had greater levels of GA_3 and smaller levels of IBA when compared to basal areas sampled (Table 1). Ross et al. (2003) concluded that in pea, mature tissues can synthesize GA such as GA_{19}. This would suggest that GA_3 applied to mature areas, as in this experiment, would be able to generate GA_3 at a similar level to apical meristem regions of the plant. However, this was not seen in the results (Fig. 4.1). The treatment with the greatest levels of GA_3 was GA_3 plus IBA (Fig 4.1). This combination differed from all other treatments by at least a 18% increase in GA_3. Transport of IBA has become clearer by labelling and inhibitor studies. A significant amount of auxin was found in root tissue that derives from shoot sources (Ross et al. 2006). However, correlation exists between root development and ability of the root to synthesize auxin (Bhalerao et al. 2002). These findings indicate IBA transport can be affected by GA_3, but this depends on the area of the plant sampled and age of the tissue.

Propagation percentage and visual rooting

Propagation studies for moroccan pincushion were conducted to determine whether the levels of GA_3 or IBA affected rooting of hormone treated vegetative cuttings. Across all treatments the successful rooting percentage was over 60% (Table 4.2). It has been reported that

exogenous GA had a positive impact on auxin signaling and transport, and thus enhances the response of *Arabidopsis* roots to exogenous auxin (Li et al. 2015). These findings combined with previous research on coral bells (Markovic and Klett 2020a), cape daisy, and mojave sage (Markovic and Klett 2020b) verify these moroccan pincushion results. Also, these results indicate a difference between drench and foliar application treatments. Successful rooting of moroccan pincushion was greater when foliar treatments were applied (Table 4.2). However, number of visible roots may help to determine the effects of hormone treatments on quantity of roots and overall quality of the rooted cutting. Number of visible roots were greatest when GA_3 alone and GA_3 plus benzyladenine were applied by drench in comparison to other treatments. GA_3 alone as a drench application had at least a 16% increase in visible roots over all other treatments.

4.2.5 Conclusion

The experiment was carried out to determine the effects of varying combinations of GA_3 with benzyladenine, ethephon, and IBA on accumulation and movement of GA_3 and IBA within moroccan pincushion stock plants. The experiment showed that among the different combinations of hormones GA_3 plus IBA had a synergistic effect and increased levels of both hormones. The application methods foliar and drench each had different results with IBA having greater levels with foliar applications and GA_3 having greater levels with drench applications. The use of GA_3 and IBA by foliar application has a potential benefit to moroccan pincushion growers due to the positive effect on propagation rate and increased IBA levels which can assist in root initiation.

Table 4.1 Mean content of GA_3 (ng/g tissue) analyzed by treatment, application method, or location within plant.

	IBA (ng/g)	**GA_3 (ng/g)**
Treatment		
Control	0.050a[z]	0.069a
GA3	0.039a	0.262b
GA3 + Auxin	1.037b	1.736c
GA3 + BA	0.201a	1.556c
GA3 + Ethephon	0.037a	0.551b
P value	0.001	0.001
Application		
Foliar	0.468b	0.382a
Drench	0.195a	1.672a
P value	0.015	0.582
Location		
Apical	0.268a	0.942a
Basal	0.331a	0.904a
P value	0.459	0.99

[z]Mean separation in columns with Tukey adjusted least squares means at $P \leq 0.05$ (lowercase letters).

Table 4.2 Treatment and application method effect on rooting percentage and number of visible roots.

Treatment	Rate ($mg \cdot L^{-1}$)	Average Rooting Percentage	Average Number of Visible Roots
control	0	97a[z]	11.7b
GA_3 Foliar	25	97a	13.4b
GA_3 + IBA Foliar	25 + 250	100a	13.3b
GA_3 + benzyladenine Foliar	25 + 250	93a	12.9b
GA_3 + ethephon Foliar	25 + 400	100a	13.2b
GA_3 Drench	25	75a	19.3d
GA_3 + IBA Drench	25 + 250	60a	12.2b
GA_3 + benzyladenine Drench	25 + 250	93a	16.6c
GA_3 + ethephon Drench	25 + 400	93a	5.4a
P value		0.874	0.025

[z]Mean separation in columns with Tukey adjusted least squares means at $P \leq 0.05$ (lowercase letters).

Figure 4.1 Mean content of GA_3 (ng/g tissue) analyzed by hormone treatment and location within moroccan pincushion. Data underwent Log transformation due to right skewness.

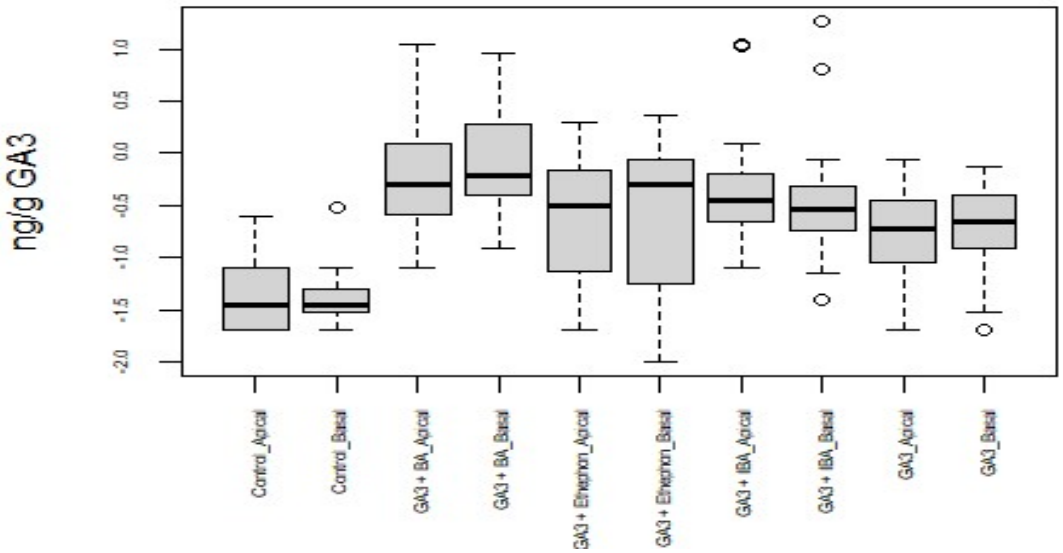


Figure 4.2 Mean content of GA_3 (ng/g tissue) analyzed by treatment and application method within moroccan pincushion. Data underwent Log transformation due to right skewness.

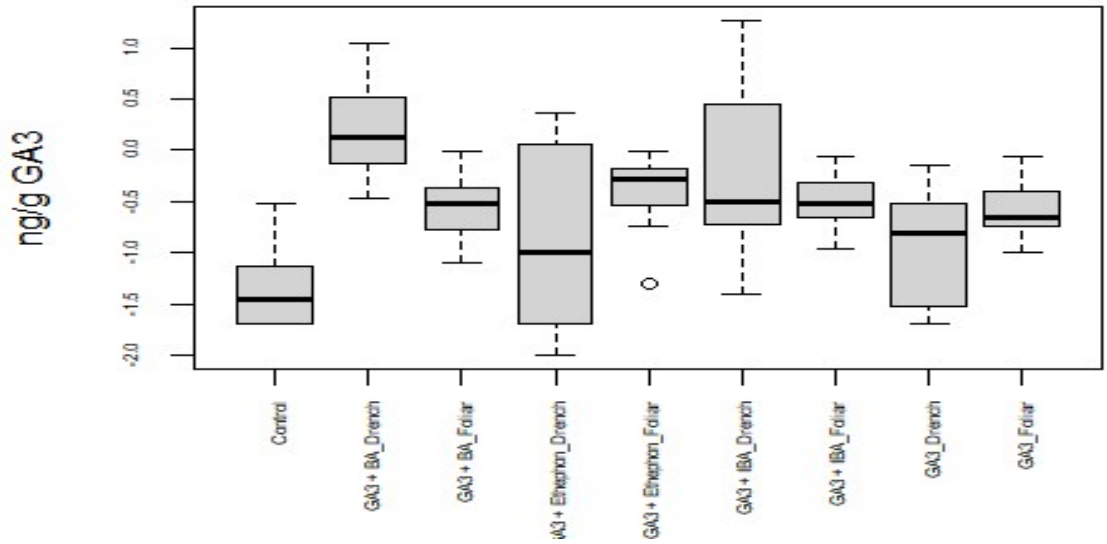


Figure 4.3 Mean content of IBA (ng/g tissue) analyzed by treatment and location within moroccan pincushion. Data underwent Log transformation due to right skewness.

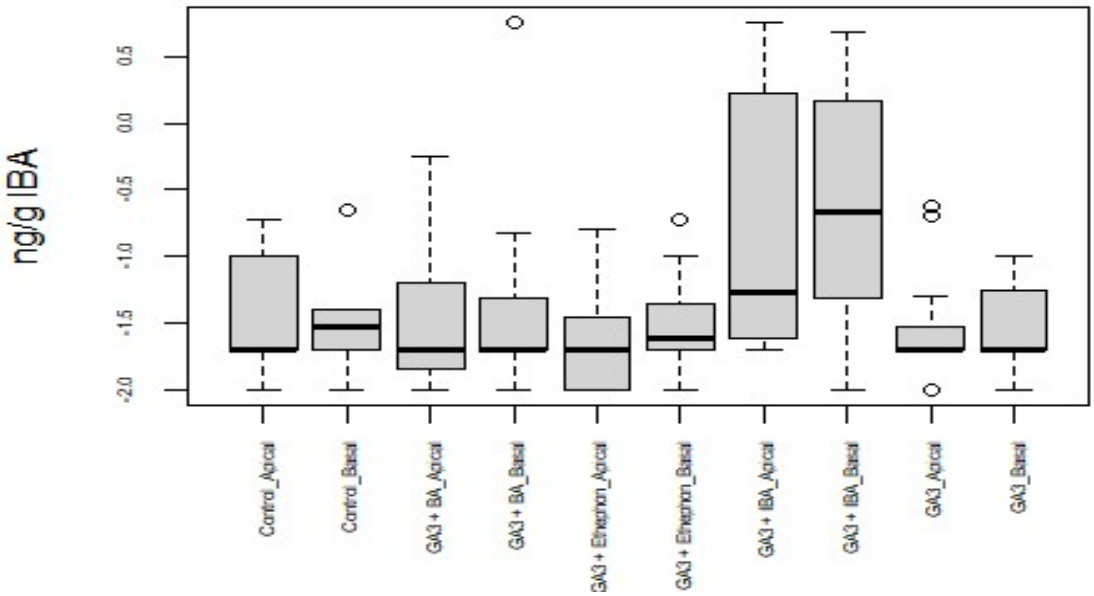
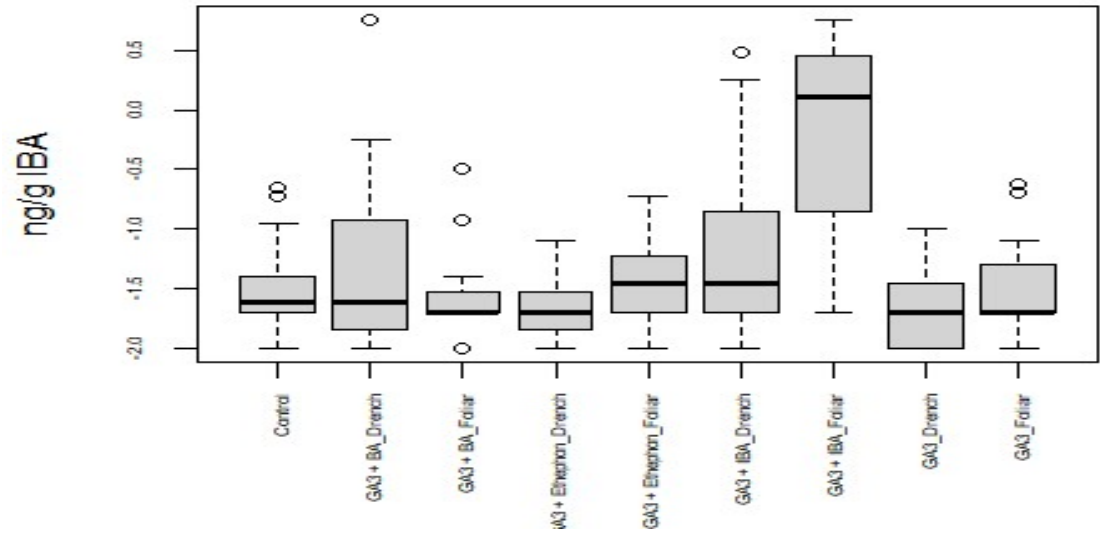


Figure 4.4 Mean content of IBA (ng/g tissue) analyzed by treatment and application method within moroccan pincushion. Data underwent Log transformation due to right skewness.



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CHAPTER 5. RESULTS & DISCUSSION

5.1. *Pterocephalus depressus* Moroccan Pincushion Leaf Nutrient Content Experiment

This chapter examines results from ICP-OES experiment for *Pterocephalus depressus* in the format of a peer-reviewed short paper journal manuscript. Results that are not included in this chapter are presented in the appendix. As defined by HortScience: Short papers must be less than 1800 words, including all titles and references. May contain two scalable images only. Images may be either pictures or tables or a combination of both. Must indicate that paper is submitted as a short paper during the submission process.

5.2. Manuscript for *Pterocephalus depressus* Leaf Nutrient Content Experiment

Plant Growth Regulator Effect on Potassium Accumulation in Stock Plant Cuttings of Moroccan Pincushion

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Subject Category: Landscape Horticulture

Plant Growth Regulator Effect on Potassium Accumulation in Stock Plant Cuttings of Moroccan Pincushion

Additional index words. propagation, *Pterocephalus depressus*, stock plant management, vegetative cuttings

5.2.1 Summary

Moroccan pincushion (*Pterocephalus depressus*) is a drought-tolerant perennial that is being used in landscapes throughout arid regions of the western United States. This paper describes an experiment researching leaf nutrient content from cuttings taken off stock plants. Moroccan pincushion stock plants received applications of gibberellic acid (GA_3), benzyladenine, ethephon, or auxin [indole-3-butyric acid (IBA)]. Plant growth regulators (PGR) were applied singularly and in combination with GA_3 to determine efficacy on stock plant growth. Each PGR treatment was also applied using either a foliar or drench application method. Several nutrients were analyzed using a nitric acid digestion of dried leaf material and then an inductively coupled plasma- optical emission spectrometer (ICP-OES) machine. The results were insignificant overall with only potassium showing differences when comparing PGR treatments. Cuttings' rooting percentage for each treatment was assessed for each nutrient accumulations in the leaf. GA_3 applied via drench applications resulted in the greatest accumulation of potassium

in moroccan pincushion cuttings. GA_3 drench treatments combined with benzyladenine and ethephon also had significant increases in cutting potassium accumulation.

5.2.2 Introduction

Production of high-quality cuttings from perennial stock plants is an important part of commercial propagation of perennial plants. Increased propagation success for moroccan pincushion will help greenhouse producers meet growing demand for the perennial groundcover. The research described in this study involves several plant growth regulators (PGR) applied to moroccan pincushion stock plants. Gibberellic acid (GA) was the focus of this research because application to perennial stock plants increased cutting production (Markovic and Klett 2020). The effects of PGR applications on cutting nutrient levels may produce a link between hormone and nutrient levels. Adventitious root formation is a complex process and nutrient levels in the cutting effect the rate at which roots form (Preece and Read 1993). One nutrient in this study, potassium, was observed promoting adventitious root formation in plants and cuttings (Zhao et al. 1991). Depletion of nutrients typically occurs during propagation (Santos et al. 2011), therefore any positive correlation between PGR treatments and increased nutrient levels would be of interest to growers. The effects of hormone levels on nutrient accumulations in vegetative cuttings used for propagation has not been thoroughly researched.

In the present work we studied the effect of four PGR on the nutrient accumulations within moroccan pincushion vegetative cuttings. The results showed that only potassium accumulation was affected by different PGR treatments.

5.2.3 Materials & Methods

Plant material and PGR treatment

Vegetative moroccan pincushion rooted cuttings were received from a local greenhouse (Gulley Greenhouse, Fort Collins, CO) and transplanted into 10 cm by 10 cm square pots containing peat substrate composed of blonde peat moss, wood fiber, dolomitic limestone, and a wetting agent (Pindstrup, Ryomgaard, Denmark). These were grown in a greenhouse with daytime temperatures monitored with an aspirator (Model M4821, Wadsworth Control Systems, Arvada, CO) sensor between 18 and 23 °C (65 and 73 °F), while night-time temperatures were held between 16 and 22 °C (61 and 73 °F). The effects of GA_3 (Valent USA Corp., Fresno, CS), auxin (IBA) (Hortus USA Corp., New York, NY), ethephon (Nufarm Americas, Inc., Alsip, IL), and benzyladenine (Fine Agrochemicals Limited, Worcester, U.K.) on hormone accumulation were assessed using plants of equal size.

Treatment applications were performed after roots were observed to strike the sides of the containers in most of the plants, about six weeks after transplant. A 3.78 L (1 gal) hand pump sprayer was used to apply GA3 (25 $mg \cdot L^{-1}$), IBA (250 $mg \cdot L^{-1}$), ethephon (400 $mg \cdot L^{-1}$), or benzyladenine (250 $mg \cdot L^{-1}$) on foliage of the whole plant. Trays with no drainage holes were filled with 3.78 L (1 gal) of each treatment and plant containers were placed in trays with 3.78 L treatment solutions for one hour to provide drench applications. Treatments were applied four times with six-week intervals in-between each application. Five replicants of each treatment were carried out.

Inductively coupled plasma optical emission spectrometry

Inductively coupled plasma optical emission spectrometry (ICP-OES) was used to determine cutting nutrient content and its effect on cutting rooting success. This analysis enabled deeper understanding of the metabolic response of the cuttings resulting from PGR treatments.

The procedure was carried out essentially as described in Reynolds et al. (2020), with the nitric acid digestion procedure from work done by Zarcinas et al. (1987). The protocol used for this analysis started with 100 mg of dried moroccan pincushion leaf tissue weighed and broken apart in a 25 mm diameter by 200 mm long glass digestion tube. Then, in the chemical hood 1 mL of trace element grade nitric acid (HNO3) was added to the dried material and a glass funnel was place on the tube to prevent evaporation. A blank sample was made by dispensing 1 mL of nitric acid into an empty digestion tube. The tubes were set in digestion blocks. The digestion program ran at a temperature of 60 °C for two hours and then 122 °C for six hours.

At the completion of the digestion program, labeled acid-resistant polypropylene15 mL tubes were used to transfer sample contents from the glass digestion tubes. Reverse osmosis treated water was added to the digest, followed by vortexing and transfer to 15 mL tubes. More water was added until the 10 mL level was reached in the test tube. The samples were stored in racks on the lab bench until analyzed using ICP-OES. The process used during ICP-OES was performed as described by Winge et al. (1978). Results were generated into an excel file and calculated based on the exact initial dry weight which was recorded while weighing the plant material from the dried samples. Nutrients analyzed during ICP-OES included: calcium, potassium, magnesium, zinc, and phosphorus.

The first set of samples were digested on October 21-23, 2019. These samples were analyzed with the ICP-OES machine November 19, 2019. The second set of samples were digested on March 5-6, 2020. These samples were analyzed with the ICP-OES machine March 16, 2020.

5.2.4 Results and Discussion

Effects of plant growth regulator application on accumulation of calcium, magnesium, zinc, and phosphorus

No significant increases in nutrient accumulations were observed for calcium, magnesium, zinc, or phosphorus for all treatments during this experiment. These nutrients have been previously connected to plant processes involved in adventitious root formation (Steffens and Rasmussen, 2016). Levels of these nutrients did not affect the successful rooting of moroccan pincushion. No significant differences observed when comparing treatments. Rooting data for moroccan pincushion was reported in a previous paper (Markovic and Klett, 2021). The levels of these nutrients that were found in the leaf tissue samples were all in large enough quantities to not be a deficiency. The use of GA, auxin, benzyladenine, or ethephon did not have a detrimental effect on accumulations of these nutrients in cuttings taken from moroccan pincushion.

Effects of plant growth regulator application on accumulation of potassium

The levels of potassium were found to have differed significantly when comparing all PGR treatments (Table 5.1). GA_3 drench at an application rate of 25 ppm resulted in the greatest accumulation of potassium in moroccan pincushion cuttings. Other significantly different treatments were drench applications of GA_3 + benzyladenine and GA_3 + ethephon (Table 5.1). The increase in potassium for three drench applications represents an interesting trend. Drench applications did not have the overall increases for other nutrients analyzed (data not shown). The effects of hormones in the root system and its effect on potassium are relatively unknown. These results are positive for helping increase rooting of moroccan pincushion through drench applications of GA_3. In another study, increased potassium availability resulted in more

adventitious root formation in cucumber, mung bean, and kidney bean (Zhao et al. 1991). The current findings indicate positive relationship between GA_3 in high concentrations in the root system and accumulation of potassium in moroccan pincushion vegetative cuttings.

Table 5.1 *Pterocephalus depressus* Moroccan pincushion mean average potassium (mg/kg) per dry leaf sample and 95% confidence intervals for each treatment. Means with different significance groups, identified by letter, are significantly different at the level of $P < 0.05$.

Treatment	Rate (ppm)	Mean Amount of Potassium	95% Confidence Interval
control-a	0	26753	(21153-32353)
benzyladenine foliar-a	250	25487	(20077-30897)
ethephon foliar-a	400	26828	(21418-32238)
GA_3 foliar-a	25	27860	(22778-32942)
GA_3 + benzyladenine foliar-a	25 + 250	28841	(23431-34251)
GA_3 + ethephon foliar-a	25 + 400	30277	(24867-35687)
GA_3 + IBA foliar-a	25 + 250	26469	(21231-31708)
GA_3 drench-d	25	51194	(45146-57243)
GA_3 + benzyladenine drench-c	25 + 250	47520	(40894-54146)
GA_3 + ethephon drench-b	25 + 400	31209	(22655-39763)
GA_3 + IBA drench-a	25 + 250	22528	(13974-31082)

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APPENDIX

A1. Chapter 1 Appendices

configure®

Ornamental Plant Growth Regulator

Active Ingredient: N-(phenylmethyl)-1H-purine-6-amine 2.0%
Other Ingredients: 98.0%
Total: 100.0%

KEEP OUT OF REACH OF CHILDREN
CAUTION

EPA Reg. No. 62097-19-82917 EPA Est. No. 39578-TX-1

NET CONTENTS: 0.5 gallon

FIRST AID

IF IN EYES:
- Hold eye open and rinse slowly and gently with water for 15-20 minutes.
- Remove contact lenses, if present, after the first 5 minutes, then continue rinsing eye.
- Call a poison control center or doctor for treatment advice.

IF SWALLOWED:
- Call a poison control center or doctor immediately for treatment advice.
- Have person sip a glass of water if able to swallow.
- Do not induce vomiting unless told to do so by a poison control center or doctor.
- Do not give anything by mouth to an unconscious person.

IF ON SKIN/CLOTHING:
- Take off contaminated clothing.
- Rinse skin immediately with plenty of water for 15-20 minutes.
- Call a poison control center or doctor for treatment advice.

Have the product container or label with you when calling a poison control center or doctor, or going for treatment. You may also contact 1-800-858-7378 (National Pesticide Information Center) for emergency medical treatment information.

FOR CHEMICAL EMERGENCY: spill, leak, fire, exposure, or accident call CHEMTREC 1-800-424-9300.

PRECAUTIONARY STATEMENTS

HAZARDS TO HUMANS AND DOMESTIC ANIMALS

CAUTION. Causes moderate eye irritation. Harmful if swallowed or absorbed through skin. Avoid contact with eyes, skin or clothing. Wash thoroughly with soap and water after handling.

PERSONAL PROTECTIVE EQUIPMENT (PPE)

Some materials that are chemical-resistant to this product are listed below. If you want more options, follow the instructions for Category C on an EPA chemical-resistance selection chart.

Applicators and other handlers must wear:
- Long-sleeved shirt and long pants.
- Chemical-resistant gloves such as barrier laminate, butyl rubber, nitrile rubber, neoprene rubber, polyvinyl chloride or viton.
- Socks and shoes.

Follow the manufacturer's instructions for cleaning/maintaining PPE. If no such instructions for washables, use detergent and hot water. Keep and wash PPE items separately from other laundry.

USER SAFETY RECOMMENDATIONS

Users should:
- Wash hands before eating, drinking, chewing gum, using tobacco or using the toilet.
- Remove clothing/PPE immediately if pesticide gets inside. Then wash thoroughly and put on clean clothing.
- Remove PPE immediately after handling this product. Wash the outside of gloves before removing. As soon as possible, wash thoroughly and change into clean clothing.

ENVIRONMENTAL HAZARDS

For terrestrial uses: Do not apply directly to water, to areas where surface water is present or to intertidal areas below the mean high water mark. Do not contaminate water when disposing of equipment wash waters or rinsate.

DIRECTIONS FOR USE

It is a violation of Federal law to use this product in a manner inconsistent with its labeling. Do not apply this product in a way that will contact workers or other persons, either directly or through drift. Only protected handlers may be in the area during application. For any requirement specific to your state or tribe, consult the agency responsible for pesticide regulation. Do not apply this product through any type of irrigation system.

AGRICULTURAL USE REQUIREMENTS

Use this product only in accordance with its labelling and with the Worker Protection Standard, 40 CFR Part 170. This Standard contains requirements for the protection of agricultural workers on farms, forests, nurseries, and greenhouses, and handlers of agricultural pesticides. It contains requirements for training, decontamination, notification and emergency assistance. It also contains specific instructions and exceptions pertaining to the statements on the label about personal protective equipment (PPE) and restricted entry intervals. The requirements in this box only apply to uses of this product covered by the Worker Protection Standard.

Do not enter or allow entry into treated areas during the restricted entry interval (REI) of 12 hours.

PPE required for early entry to treated areas that is permitted under the Worker Protection Standard and that involves contact with anything that has been treated such as, plants, soil or water is:
- Coveralls
- Shoes plus socks
- Chemical-resistant gloves such as barrier laminate, butyl rubber, nitrile rubber, neoprene rubber, polyvinyl chloride or viton

GENERAL INFORMATION

Do not apply this product through any type of irrigation system.

FOR USE IN: COMMERCIAL GREENHOUSES, GLASSHOUSES (WHERE PLANTS ARE GROWN IN CONTAINERS), AND ON ACTIVELY GROWING STOCK PLANTS

CONFIGURE increases lateral branching and promotes flowering in certain ornamental crops. In addition, applications of CONFIGURE reduce the overall height of the plant resulting in more compact and marketable plants.

Plant response to CONFIGURE is strongly influenced by cultural and environmental variables. Growing media, water/fertilizer management, temperature, light, greenhouse composition, and other cultural practices may impact plant response. Plant size, container size and cultivar or variety can also influence treatment response. Although multiple cultivars have been tested for sensitivity to CONFIGURE and have demonstrated high levels of tolerance, it is impossible to insure an acceptable response in all cultivars. **First time users of CONFIGURE should first conduct trials on a limited number of plants to determine crop response.**

Figure A1.1 Configure PGR Label

ProGibb® T&O

PLANT GROWTH REGULATOR (PGR) SOLUTION

FOR ORGANIC PRODUCTION

For use on turf and ornamental crops.

ACTIVE INGREDIENT:

Gibberellic Acid	4.0% w/w
OTHER INGREDIENTS	96.0% w/w
TOTAL	100.0% w/w

ProGibb T&O liquid contains approximately 1.0 gram active ingredient per fluid ounce of formulated product.

EPA Reg. No. 73049-15
EPA Est. No. 33762-IA-001 List No. 22055

INDEX:

1.0 First Aid
2.0 Precautionary Statements
- 2.1 Hazards to Humans and Domestic Animals
- 2.2 Personal Protective Equipment (PPE)
- 2.3 User Safety Recommendations
- 2.4 Environmental Hazards
- 2.5 Physical or Chemical Hazards

3.0 Directions for Use
4.0 Agricultural Use Requirements
5.0 Non-Agricultural Use Requirements
6.0 Product Information
7.0 General Instructions
8.0 Determining Optimal Application Rates
- 8.1 Limitations

9.0 Mixing Instructions and Rate Conversion Table
- 9.1 Rate Conversion Table

10.0 Ornamental Crops, Cut Flowers and Turfgrass
- 10.1 Spray Guidelines for Ornamentals
- 10.2 Applications to Cut Flowers
- 10.3 Bedding Plants, Annual and Perennial Potted Crops, Field-Grown Ornamentals and Bulb Crops
- 10.4 Applications to Turfgrass

11.0 Storage and Disposal
12.0 Notice to User

KEEP OUT OF REACH OF CHILDREN

WARNING – AVISO

Si usted no entiende la etiqueta, busque a alguien para que se la explique a usted en detalle. (If you do not understand the label, find someone to explain it to you in detail).

For MEDICAL and TRANSPORT Emergencies ONLY Call 24 Hours A Day 1-800-892-0099. For All Other Information Call 1-800-89-VALENT (898-2536).

1.0 FIRST AID

If in eyes	• Hold eye open and rinse slowly and gently with water for 15-20 minutes. • Remove contact lenses, if present, after the first 5 minutes, then continue rinsing eye. • Call a poison control center or doctor for treatment advice.
If swallowed	• Call a poison control center or doctor immediately for treatment advice. • Have person sip a glass of water if able to swallow. • Do not induce vomiting unless told to do so by the poison control center or doctor. • Do not give anything by mouth to an unconscious person.
If inhaled	• Move person to fresh air. • If person is not breathing, call 911 or an ambulance, then give artificial respiration, preferably by mouth-to-mouth, if possible. • Call a poison control center or doctor for further treatment advice.
If on skin or clothing	• Take off contaminated clothing. • Rinse skin immediately with plenty of water for 15-20 minutes. • Call a poison control center or doctor for treatment advice.

HOT LINE NUMBER

Have the product container or label with you when calling a poison control center or doctor, or going for treatment. For medical emergencies, you may also call toll-free 1-800-892-0099 for treatment information.

2.0 PRECAUTIONARY STATEMENTS

2.1 HAZARDS TO HUMANS AND DOMESTIC ANIMALS

WARNING

Causes substantial but temporary eye injury. Harmful if inhaled or absorbed through skin. Do not get in eyes or on clothing. Avoid breathing vapor or spray mist, and avoid contact with skin. Wash thoroughly with water and soap after handling. Remove and wash contaminated clothing before reuse.

2.2 PERSONAL PROTECTIVE EQUIPMENT (PPE)

Some materials that are chemical-resistant to this product are listed below. If you want more options, follow the instructions for Category C on an EPA chemical resistance category selection chart.

Applicators and other handlers must wear:

- Long sleeved shirt
- Long pants
- Chemical resistant gloves, such as barrier laminate, butyl rubber, nitrile rubber, neoprene rubber, polyvinyl chloride, and viton
- Shoes plus socks
- Protective eyewear

Discard clothing and other absorbent materials that have been drenched or heavily contaminated with this product's concentrate. Do not reuse them.

Follow manufacturer's instructions for cleaning/maintaining PPE. If no such instructions for washables, use detergent and hot water. Keep and wash PPE separately from other laundry.

CONTINUED

Figure A1.2 ProGibb PGR Label

Verve™

Plant Growth Regulator

Intented for Commercial or Agricultural Use Only

For use on Apples, Blackberries, Blueberries, Cantaloupes, Cherries, Grapes, Peppers, Tobacco, Field and Greenhouse Tomatoes, Walnuts, and for Minimizing Lodging in Barley and Wheat.

For the Removal of Dwarf Mistletoe in Ornamental Conifers and Leafy Mistletoe in Ornamental Deciduous Trees, for the Elimination of Undesirable Fruit on Ornamental Trees and Shrubs, for Inducing Flowering of Ornamental Bromeliads, for Increased Lateral Branching in Ornamentals, for Reducing Plant Height of Potted Daffodils and Stem Topple of Potted Hyacinths, in the Production of Cucumber, Squash and Pumpkin Hybrid Seed, and for Use on Turf including Golf Courses and Sod Farms.

ACTIVE INGREDIENT:	
Ethephon: (2-Chloroethyl) phosphonic acid*	21.7%
OTHER INGREDIENTS:	78.3%
TOTAL:	100.0%

*1 Gallon contains 2 lb ethephon.

KEEP OUT OF REACH OF CHILDREN

DANGER / PELIGRO

Si usted no entiende la etiqueta, busque a alguien para que se la explique a usted en detalle.
(If you do not understand the label, find someone to explain it to you in detail.)

See Inside Label Booklet for PRECAUTIONARY STATEMENTS

For Chemical Spill, Leak, Fire, or Exposure, Call CHEMTREC (800) 424-9300.
For Medical Emergencies Only, Call (877) 325-1840.

FIRST AID	
IF IN EYES	• Hold eye open and rinse slowly and gently with water for 15-20 minutes. • Remove contact lenses, if present, after the first 5 minutes, then continue rinsing eye. • Call a poison control center or doctor for treatment advice.
IF SWALLOWED	• Call a poison control center or doctor immediately for treatment advice. • Have person sip a glass of water if able to swallow. • Do not induce vomiting unless told to do so by the poison control center or doctor. • Do not give anything to an unconscious person.
IF ON SKIN OR CLOTHING	• Take off contaminated clothing. • Rinse skin immediately with plenty of water for 15-20 minutes. • Call a poison control center or doctor for treatment advice.
IF INHALED	• Move person to fresh air. • If person is not breathing, call 911 or an ambulance, then give artificial respiration, preferably by mouth-to-mouth, if possible. • Call a poison control center or doctor for further treatment advice.

HOTLINE NUMBER

Have the product container or label with you when calling a poison control center or doctor, or going for treatment. You may also contact (877) 325-1840 for emergency medical treatment information.

NOTE TO PHYSICIAN

Probable mucosal damage may contraindicate the use of gastric lavage. No specific antidote is available. All treatments should be based on observed signs and symptoms of distress in the patient. Overexposure to materials other than this product may have occurred.
Victims of severe overexposure by inhalation should be kept under medical observation for up to 72 hours for delayed onset of pulmonary edema. In a victim of overexposure by ingestion, careful gastric lavage is required due to the possibility of stomach or esophageal perforation. This material is an acid but the use of alkaline substances to neutralize it is contraindicated.

EPA REG. NO. 228-660

Manufactured for
Nufarm Americas Inc.
11901 S. Austin Avenue
Alsip, IL 60803

Nufarm Grow a better tomorrow.

Figure A1.3 Verve PGR Label

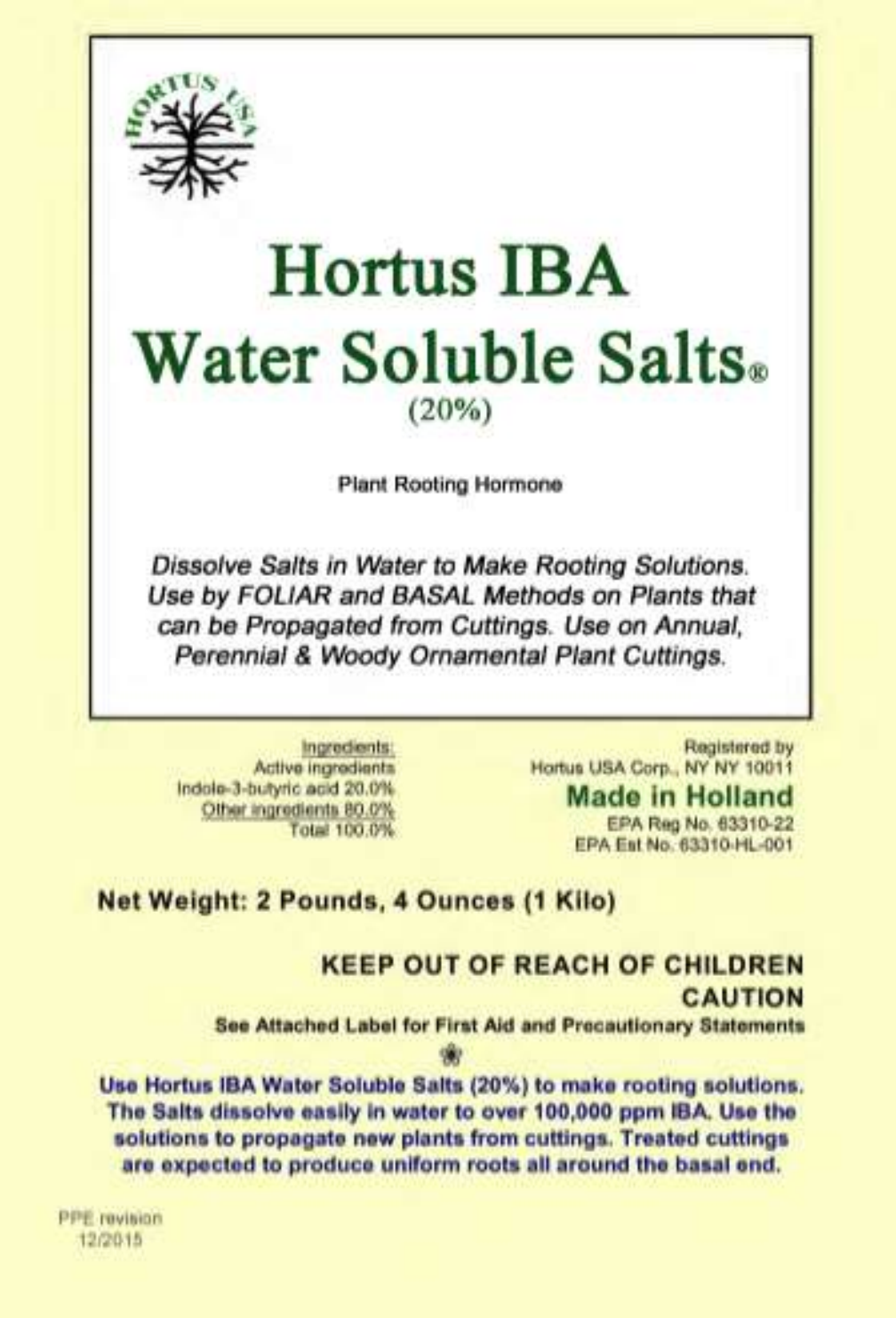

Figure A1.4 Auxin (IBA Water Soluble Salts) PGR Label

A2. Chapter 2 Appendices

A3. Chapter 3 Appendices

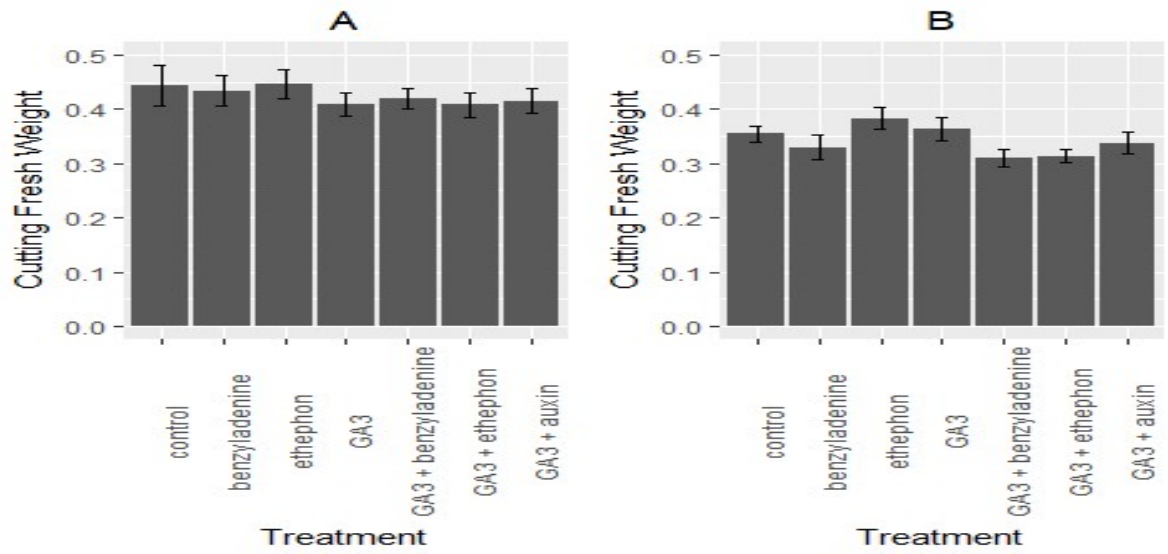

Fig. A3.1. Mean average fresh weight per cutting from moroccan pincushion stock plants averaged over four harvest dates for Expt. 1 (A) and Expt. 2 (B) as influenced by seven plant growth regulator treatments: benzyladenine, ethephon, gibberellic acid 3 (GA_3), GA_3 + benzyladenine, GA_3 + ethephon, GA_3 + auxin, and control.

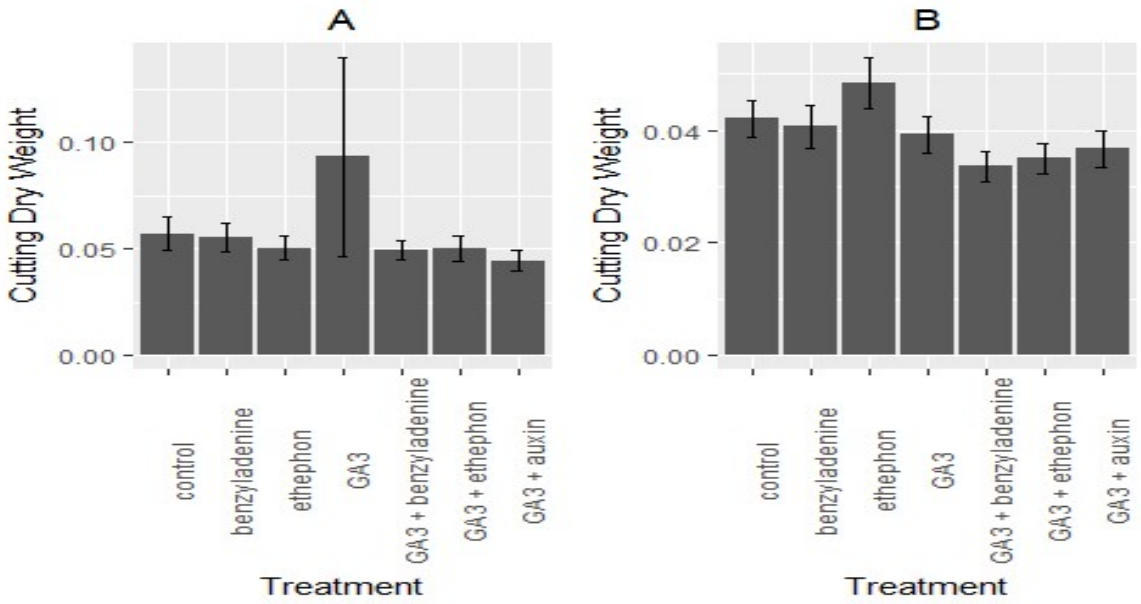

Fig. A3.2. Mean average dry weight per cutting from moroccan pincushion stock plants averaged over four harvest dates for Expt. 1 (A) and Expt. 2 (B) as influenced by seven plant growth regulator treatments: benzyladenine, ethephon, gibberellic acid 3 (GA_3), GA_3 + benzyladenine, GA_3 + ethephon, GA_3 + auxin, and control.

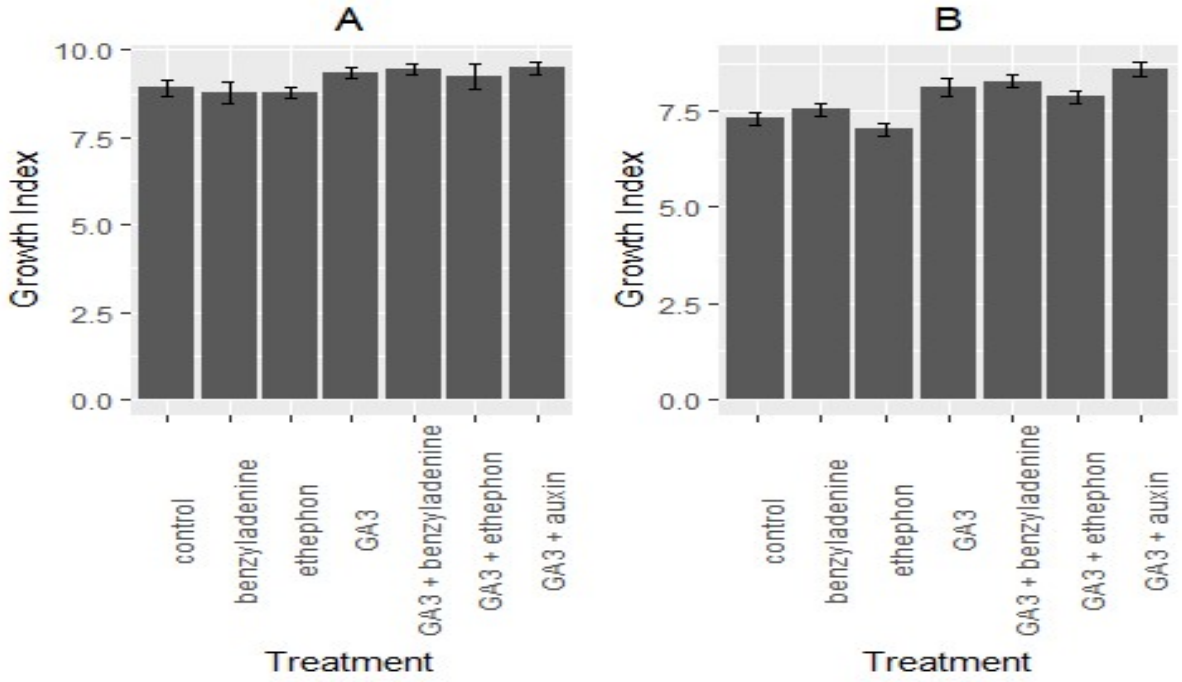

Fig. A3.3. Mean average growth index per plant from moroccan pincushion stock plants averaged over four harvest dates for Expt. 1 (A) and Expt. 2 (B) as influenced by seven plant growth regulator treatments: benzyladenine, ethephon, gibberellic acid 3 (GA_3), GA_3 + benzyladenine, GA_3 + ethephon, GA_3 + auxin, and control.

Table A3.1. *Pterocephalus depressus* Moroccan pincushion Exp. 1 mean average number of cuttings per plant and 95% confidence intervals for each treatment. Means with different significance groups are significantly different at the level of $P < 0.05$.

Treatment	Average no. Cuttings per Plant	Lower CI 2.5%	Upper CI 97.5%	Significance Group
control	7.94	6.66	9.23	1
benzyladenine_Foliar	9.69	8.38	10.99	12
ethephon_Foliar	8.34	7.04	9.65	12
GA3_Foliar	10.02	8.91	11.14	12
GA3 + benzyladenine_Foliar	11	9.89	12.11	2
GA3 + ethephon_Foliar	10.32	9.15	11.48	12
GA3 + auxin_Foliar	9.88	8.76	10.99	12

Table A3.2. *Pterocephalus depressus* Moroccan pincushion Exp. 2 mean average number of cuttings per plant and 95% confidence intervals for each treatment. Means with different significance groups are significantly different at the level of $P < 0.05$.

Treatment	Average no. Cuttings per Plant	Lower CI 2.5%	Upper CI 97.5%	Significance Group
control	5.63	4.81	6.47	12
benzyladenine_Foliar	7.22	6.28	8.16	123
ethephon_Foliar	5.33	4.41	6.26	1
GA3_Foliar	6.91	6.07	7.74	123
GA3 + benzyladenine_Foliar	8.13	7.31	8.96	3
GA3 + ethephon_Foliar	7.38	6.55	8.22	23
GA3 + auxin_Foliar	7.77	6.94	8.61	3

Table A3.3. *Pterocephalus depressus* Moroccan pincushion Exp. 1 mean average final dry weight per plant and 95% confidence intervals for each treatment. Means with different significance groups are significantly different at the level of $P < 0.05$.

Treatment	Final Dry Weight (g)	Lower CI 2.5%	Upper CI 97.5%	Significance Group
control	1.424	1.234	1.613	2
benzyladenine_Foliar	1.367	1.142	1.592	2
ethephon_Foliar	1.352	1.125	1.575	2
GA3_Foliar	1.241	1.052	1.431	12
GA3 + benzyladenine_Foliar	1.324	1.111	1.489	2
GA3 + ethephon_Foliar	1.324	1.134	1.513	2
GA3 + auxin_Foliar	1.453	1.264	1.642	2
GA3_Drench	0.942	0.591	1.289	12
GA3 + benzyladenine_Drench	1.141	0.791	1.489	12
GA3 + ethephon_Drench	1.181	0.831	1.529	12
GA3 + auxin_Drench	0.575	0.185	0.965	1

Table A3.4. *Pterocephalus depressus* Moroccan pincushion Exp. 2 mean average final dry weight per plant and 95% confidence intervals for each treatment. Means with different significance groups are significantly different at the level of $P < 0.05$.

Treatment	Final Dry Weight (g)	Lower CI 2.5%	Upper CI 97.5%	Significance Group
control	2.74	2.018	3.47	3
benzyladenine_Foliar	2.79	2.068	3.52	3
ethephon_Foliar	2.55	1.826	3.27	23
GA3_Foliar	3.17	2.526	3.82	3
GA3 + benzyladenine_Foliar	3.35	2.699	3.99	3
GA3 + ethephon_Foliar	3.19	2.516	3.86	3
GA3 + auxin_Foliar	3.11	2.466	3.76	3
GA3_Drench	2.85	1.596	4.11	23
GA3 + benzyladenine_Drench	0.36	-0.761	1.48	12
GA3 + ethephon_Drench	1.11	-0.347	2.55	123
GA3 + auxin_Drench	0	-1.121	1.12	1

Table A3.5. *Pterocephalus depressus* Moroccan pincushion Exp. 1 mean average root rating (1-5) and 95% confidence intervals for each treatment. Means with different significance groups are significantly different at the level of $P < 0.05$.

Treatment	Root Rating (1-5)	Lower CI 2.5%	Upper CI 97.5%	Significance Group
control	3.94	3.593	4.29	5
benzyladenine_Foliar	3.83	3.419	4.25	45
ethephon_Foliar	3.92	3.502	4.33	45
GA3_Foliar	3.12	2.769	3.47	34
GA3 + benzyladenine_Foliar	3.18	2.828	3.52	345
GA3 + ethephon_Foliar	2.82	2.475	3.17	23
GA3 + auxin_Foliar	3.88	3.534	4.23	45
GA3_Drench	2.01	1.357	2.64	123
GA3 + benzyladenine_Drench	1.81	1.157	2.44	12
GA3 + ethephon_Drench	2.83	2.157	3.44	2345
GA3 + auxin_Drench	1.00	0.282	1.72	1

Table A3.6. *Pterocephalus depressus* Moroccan pincushion Exp. 2 mean average root rating and 95% confidence intervals for each treatment. Means with different significance groups are significantly different at the level of $P < 0.05$.

Treatment	Root Rating (1-5)	Lower CI 2.5%	Upper CI 97.5%	Significance Group
control	3.92	3.374	4.462	3
benzyladenine_Foliar	3.53	2.957	4.043	3
ethephon_Foliar	3.17	2.624	3.712	3
GA3_Foliar	3.33	2.848	3.819	3
GA3 + benzyladenine_Foliar	3.93	3.448	4.419	3
GA3 + ethephon_Foliar	2.86	2.354	3.361	3
GA3 + auxin_Foliar	3.67	3.181	4.152	3
GA3_Drench	2.25	1.309	3.191	23
GA3 + benzyladenine_Drench	0.43	-0.441	1.241	12
GA3 + ethephon_Drench	2.01	0.914	3.086	123
GA3 + auxin_Drench	0.00	-0.841	0.841	1

A4. Chapter 4 Appendices

A5. ICP-OES Statistics

A5.1 SOP for ICP-OES performed

ICP-OES protocol 2013

- Connect tubes to peristaltic pump, one for waste (red) closest to the ICP, one for sample (black). Wedge both ends into the notches, place tubes in their grooves and close clamp in back, control tightness if needed with screws. Note: the pump turns clockwise, so the side of the sample tube coming from the autosampler should be in the front, and the waste tube coming from the machine too
- Clamp tubes in back of autosampler into place as well (no need to tighten). There are two parts: the curved white part comes up from the front, then the yellow piece clicks over it from the back. You may have to pull the little white ring next to the yellow part a bit to make it click
- Gas tanks for nitrogen and argon are normally kept open. Check the gas tank pressures for N2 and argon – pressure normally is 100 psi on both (should be at least 60). Check the tank is still full enough, try to have it at least a quarter, ideally half full for a long run (little gauge in center of tank)
- Check that the water rinse level is high enough (carboy below with funnel) and waste tank has space
- Open program WinLab32 on computer, if not already open
- Under **System** – go to **pump on/off** and click **on**
- Under **Analysis** – go to **Autosampler** – **probe up/down** – **down**
- Check liquids are running in both sample tube and waste tube (waste tube looks like pearly droplets). If needed fill up sipper reservoir with water from squeeze bottle
- Under **system** click **plasma on/off**. It takes a minute for the torch to light, and the pump will turn off and on during this process
- Check you see the flame behind the ICP window
- Under **file** – **open** – **method** - choose **Smits4** as method
- Under **tools** – **open sample info editor** – a table will open
- Place your tubes on the autosampler. The standards and QC go in the big holes on the autosampler. These are numbered 1-10. Use 50 mL tubes for these. The blank goes in the back, then Std1, Std2 and QC (see below for what is in them). The samples go in racks, and are numbered autosampler positions 11-… There are different racks you can use, for smaller (8 mL) or bigger (15 mL) tubes. The total # of samples you can do using the 8 mL racks is 360 (4 x 90) and with the 15 mL racks 240 (4 x 60). Under **Options** – **Autosampler** – you can browse between racks. Choose either the name that ends in 360 (if you use the 8 mL

tubes) or the one that ends in 240 (for the 15 mL tubes). There should not be open spaces between samples. You may want to put water or QC1 as samples now and then (every 50 or between sample sets or between high and low expected concentrations)

- **Fill out the autosampler sample info table.** In the row where it says sample 1, type in the column autosampler location (AS location) 11. Sample 1 is 12, etc. Under Sample ID type the names of all the samples (keep it simple, so e.g. A1-A40). It helps to **use the autofill feature** in the table: right click on table cell, choose column fill. Then in the window that opens select the autosampler nr range and the sample nr range and OK. You can do this for the sample nr and for the sample ID (next column). *Note:* if the program says the max nr of samples is 250 you can change it to 360 by clicking edit – append rows – and change the max # of samples. **Save as sample info file** using e.g., the date as file name (080613 for instance).
- [If you need to make a new design: Under utilities – data manager – report – create new design – next – today only – next – report format: crosstabshortlandscape next – click date and time – next – save as e.g. Smits4 and a letter]
- If using an existing design: Under **Auto** icon open **automated analysis control** (if not already open)
- Open the sample info file of the day (if not already open)
- Click on **open** under **results data** set name, type a name e.g. the date, and click ok
- Set the machine to auto shutdown if it will run past 5 pm. Select what time it should shut down (including plasma and pump), and what time it has to turn back on (<u>not</u> plasma and pump)
- Click **analyze**. You will now see a list of your Stds and samples
- Check the sample list. If not complete, click **rebuild list**
- If this is the first run of the day, click Hg auto wavelength realign box
- Hit **analyze all** icon on top of that window
- Click on results icon to show progress, click on spectra icon to show spectra
- Stay around and check all goes well until the machine passed the QC and starts on the samples
- Check on machine every 90 min or so, look at results window and spectra window. Refill the QC (which is the same solution as standard 1). In the analysis history it should say analyzed for the samples and QC passed for the QC (every 20 samples).
- *When it is done*
- Under **file – utilities – data manager – Library category – choose results – ok**
- Select your file of the day (name = date usually)
- Click **report**
- Choose **existing design – browse – smits4 – next – select all**
- Click **Preview - print**
- Click **download button** (envelope with down arrow)
- Choose **MS Excel 97-2000 data only**, and choose disk file to save it to
- Click **OK**

- Choose flash drive (E) on computer and **save**
- Open it with Excel from flash drive to check
- If machine is done for the day make sure torch is off, tubes are loose.

Standards for new ICP-OES (July 10 2013 - present)

Calibration Blank (5% HNO3)

- ***STD 1*** **2 ppm Cd, Cr, Cu, Mn, Mo, Ni, Zn, Fe, Pb**

  & 1 ppm Se, As, W

  In blank

- ***STD 2*** **10 ppm S, Mg, Ca, V, P, K**

  In blank

How to make new standards:

Make 1 L blank: 50 mL HNO_3 in 950 mL dd water.

Fill volumetric 500 mL flask (bulb with neck) with ~400 mL blank.

Add each element to flask using 1 mL pipette (blue tip) from "holy stocks" solutions from the Soil, Water and Plant Testing lab. Most are 1,000 ppm, but some are 10,000 ppm so check!). For example: 1000 ppm → 2 ppm is 500x dilution, so add 1 mL per 500 mL.

Fill flask up to the mark with more blank. Mix well and transfer to standard bottle.

A5.2 Tables and Figures from ICP-OES

Table A5.1 *Pterocephalus depressus* Moroccan pincushion mean average phosphorus (mg/kg) per dry leaf sample and 95% confidence intervals for each treatment. Means with different significance groups are significantly different at the level of $P < 0.05$.

Treatment	Mean Amount of Phosphorus	Lower CI 2.5%	Upper CI 97.5%	Significance Group
control	3964	3213	4715	1
Benzyladenine Foliar	3837	3111	4562	1
Ethephon Foliar	4364	3638	5089	1
GA3 Foliar	3895	3214	4577	1
GA3 + benzyladenine Foliar	3616	2890	4342	1
GA3 + ethephon Foliar	3969	3243	4695	1
GA3 + IBA Foliar	4413	3711	5116	1
GA3 Drench	4612	3800	5423	1
GA3 + benzyladenine Drench	4175	3286	5063	1
GA3 + ethephon Drench	3711	2563	4858	1
GA3 + IBA Drench	3538	2391	4685	1

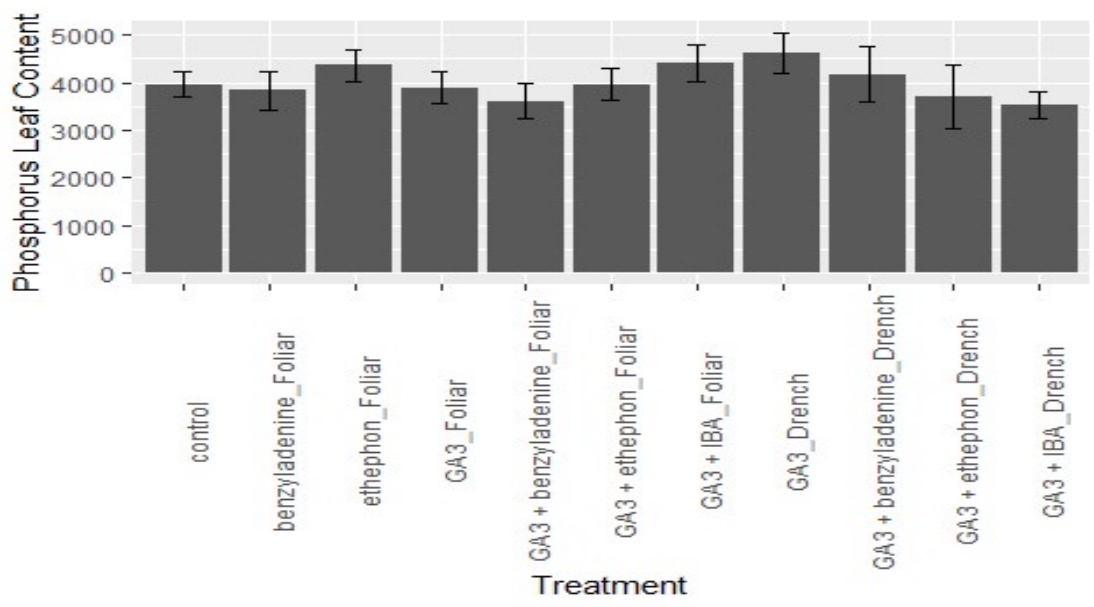

Fig. A5.1. *Pterocephalus depressus* Moroccan pincushion mean average phosphorus (mg/kg) per dry leaf sample. Standard error bars indicate a 95% confidence interval for the mean.

Table A5.2 *Pterocephalus depressus* Moroccan pincushion mean average sodium (mg/kg) per dry leaf sample and 95% confidence intervals for each treatment. Means with different significance groups are significantly different at the level of $P < 0.05$.

Treatment	Mean Amount of Sodium	Lower CI 2.5%	Upper CI 97.5%	Significance Group
control	845	620	1069	1
Benzyladenine Foliar	970	753	1187	1
Ethephon Foliar	965	748	1182	1
GA3 Foliar	536	332	740	1
GA3 + benzyladenine Foliar	754	537	971	1
GA3 + ethephon Foliar	696	479	913	1
GA3 + IBA Foliar	688	478	899	1
GA3 Drench	716	473	959	1
GA3 + benzyladenine Drench	717	451	983	1
GA3 + ethephon Drench	989	646	1332	1
GA3 + IBA Drench	666	323	1009	1

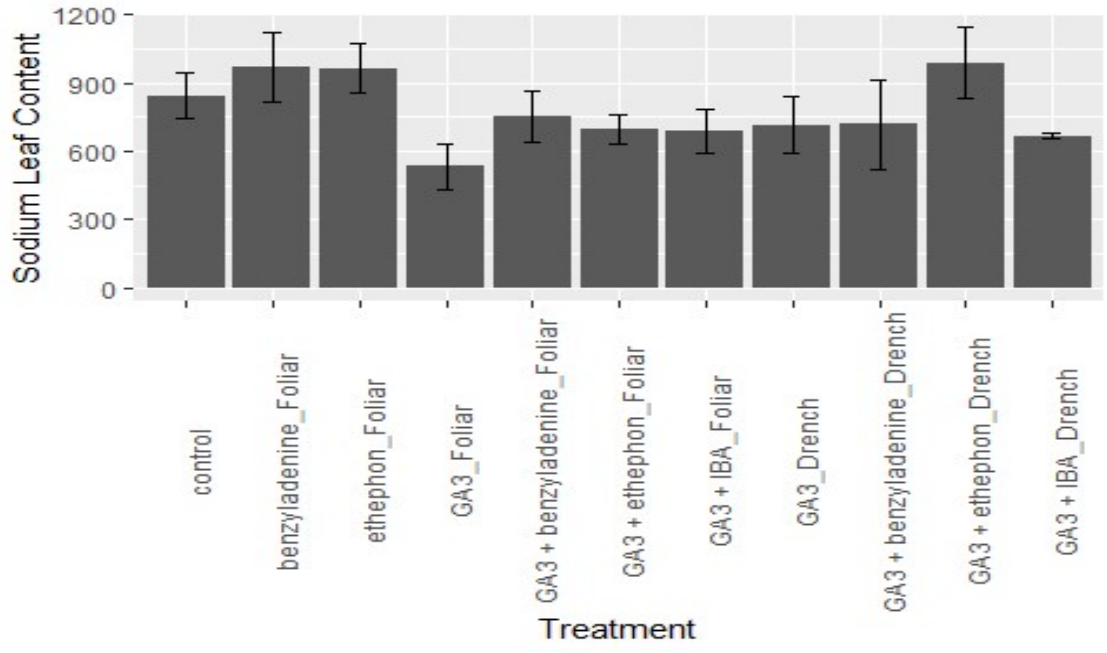

Fig. A5.2. *Pterocephalus depressus* Moroccan pincushion mean average sodium (mg/kg) per dry leaf sample. Standard error bars indicate a 95% confidence interval for the mean.

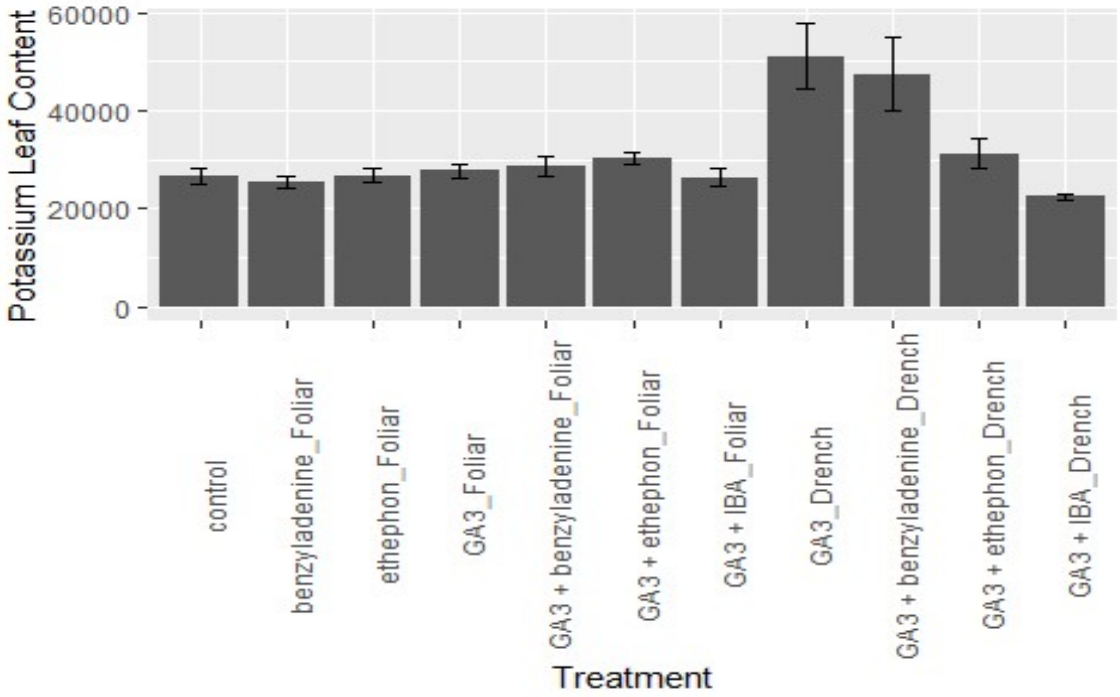

Fig. A5.3. *Pterocephalus depressus* Moroccan pincushion mean average potassium (mg/kg) per dry leaf sample. Standard error bars indicate a 95% confidence interval for the mean.

Table A5.3 *Pterocephalus depressus* Moroccan pincushion mean average calcium (mg/kg) per dry leaf sample and 95% confidence intervals for each treatment. Means with different significance groups are significantly different at the level of $P < 0.05$.

Treatment	Mean Amount of Calcium	Lower CI 2.5%	Upper CI 97.5%	Significance Group
control	10117	8435	11800	1
benzyladenine Foliar	10121	8496	11747	1
ethephon Foliar	10819	9194	12445	1
GA3 Foliar	8800	7274	10327	1
GA3 + ethephon Foliar	8645	7019	10270	1
GA3 + benzyladenine Foliar	9291	7665	10916	1
GA3 + IBA Foliar	10021	8447	11595	1
GA3 Drench	11466	9649	13284	1
GA3 + benzyladenine Drench	9780	7789	11771	1
GA3 + ethephon Drench	8157	5587	10727	1
GA3 + IBA Drench	8725	6155	11295	1

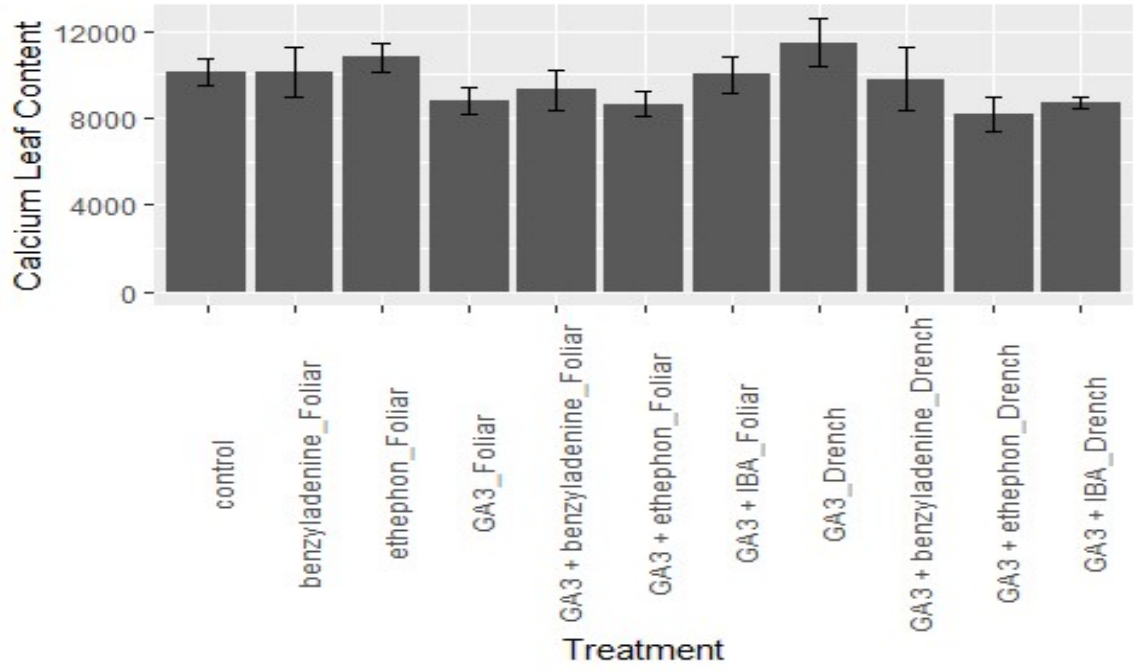

Fig. A5.4. *Pterocephalus depressus* Moroccan pincushion mean average calcium (mg/kg) per dry leaf sample. Standard error bars indicate a 95% confidence interval for the mean.

Table A5.4 *Pterocephalus depressus* Moroccan pincushion mean average magnesium (mg/kg) per dry leaf sample and 95% confidence intervals for each treatment. Means with different significance groups are significantly different at the level of $P < 0.05$.

Treatment	Mean Amount of Magnesium	Lower CI 2.5%	Upper CI 97.5%	Significance Group
control	3648	3225	4072	1
benzyladenine Foliar	3671	3261	4080	1
ethephon Foliar	3712	3302	4121	1
GA3 Foliar	3357	2972	3741	1
GA3 + benzyladenine Foliar	3211	2801	3620	1
GA3 + ethephon Foliar	3324	2914	3733	1
GA3 + IBA Foliar	3451	3055	3848	1
GA3 Drench	4044	3586	4501	1
GA3 + benzyladenine Drench	3693	3192	4195	1
GA3 + ethephon Drench	3421	2774	4068	1
GA3 + IBA Drench	2930	2282	3577	1

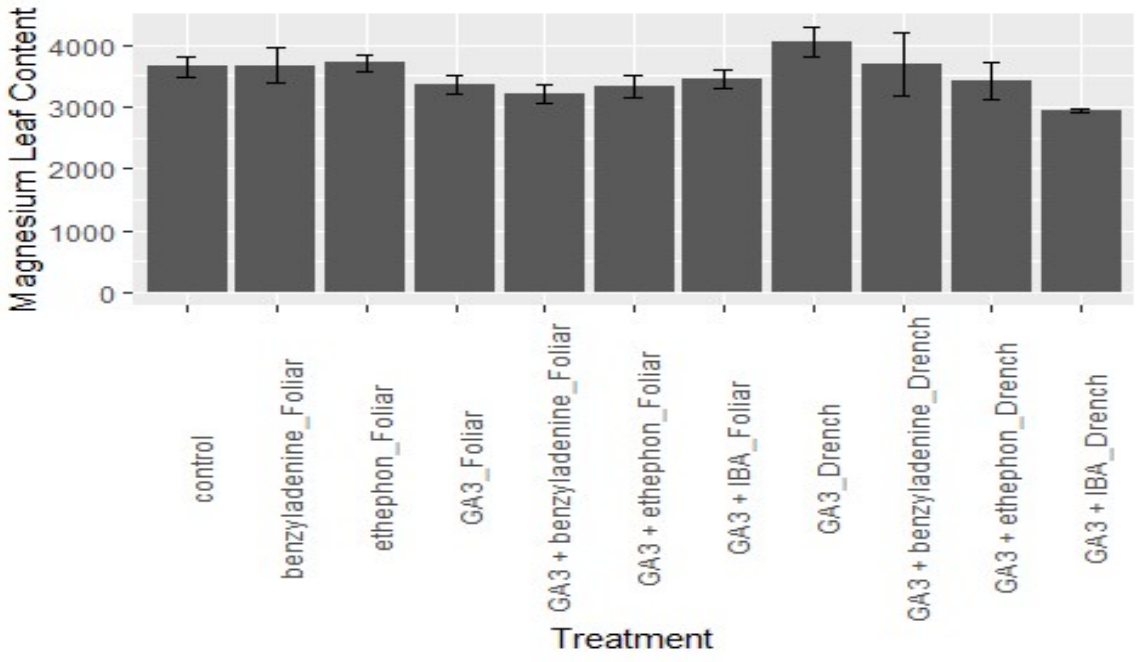

Fig. A5.5. *Pterocephalus depressus* Moroccan pincushion mean average magnesium (mg/kg) per dry leaf sample. Standard error bars indicate a 95% confidence interval for the mean.