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In vitro propagation of a critically endangered African violets (Saintpaulia rupicola B.L. Burtt)

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In vitro propagation of a critically endangered African violet (*Saintpaulia rupicola* B.L. Burtt)

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Abstract

The *in vitro* propagation is a promising method for the production of plants in species that are endangered, commercially valuable, and/or otherwise difficult to propagate through traditional horticultural methods. Leaf and petiole explants of critically endangered African violets (*Saintpaulia rupicola* B.L. Burtt) were cultured on a chemically defined medium that was supplemented with six different concentrations of thidiazuron (TDZ). The concentrations were used for either an induction medium on which the explants remained throughout development, or an induction medium on which the explants remained for 10 days followed by a basal growth medium for the remainder of the 12 weeks. None of the petiole explants grew or developed any organs. Leaf explants from all treatments developed callus from which adventitious shoots differentiated. Initially leaf explants with 5 µM TDZ preformed significantly better than all treatments apart from 8 µM TDZ. Upon further experimentation, TDZ concentrations lower than 5 µM had no significant difference from the 5µM TDZ. Callus isolated from leaf explants was placed on a basal medium and subsequently formed adventitious shoots. Isolated shoots were able to grow in size and develop further when planted on a basal medium or directly on soil. Isolated shoot tips were also encapsulated in sodium alginate to form synthetic seeds, or synseeds. Experiments were performed to determine if 2% or 3% sodium alginate was better for the emergence of the isolated shoots. Experiments were then performed with 2% sodium alginate synseeds with four concentrations of plant preservative mixture^{TM} (PPM^{TM}). There was no significant difference between the PPM^{TM} treatments so the largest concentration, 5.0 ml/L, was used for remaining experiments. Finally, experiments were performed with 0.0 ml/L and

5.0 ml/L PPM^{TM} included in the matrix of synseeds planted on sterile vermiculite, non-sterile vermiculite, and non-sterile soil. Synseeds planted on non-sterile soil had significantly less plant growth than all other media treatments. All experiments with synseeds had shoots emerge that then rooted and grew into new plants. The methods presented in this thesis are useful in the mass production of new *S. rupicola* plants for the purposes of conservation both *ex situ* and *in situ*.

Introduction

Saintpulia rupicola B.L. Burtt is a critically endangered species of African violet that is endemic to Kenya, specifically the Cha Simba and Mwarakaya regions (Eastwood et al. 1998). Populations from each region, one from each, has no more than 100 individuals (Eastwood et al. 1998). *Saintpaulia rupicola* is critically endangered because of its sensitivity to drought (Johansson 1978), and the encroachment of agriculture with resultant tree felling (Eastwood et al. 1998) is removing the canopy needed for the plants to get optimal levels of light and decreasing the moisture available.

African violets are known to be unsuccessful at reproduction in nature. This may be the result of population fragmentation that reduces pollinator service (Kolehamainen and Mutikainen 2006). However, African violets that are pollinated commonly abort their flowers or fertilized fruits (Kolehamainen and Mutikainen 2006). This regular abortion of flowers and fruits, especially fruits from fertilized flowers, and limited pollinator service, greatly decreases the regeneration of African violets in the wild. Limited regeneration means that investigations into the use of alternative reproductive methods, such as *in vitro* propagation, are prudent to the survival of the species.

There are many *in vitro* techniques used for the purpose of propagation. Among these are caulogenesis and somatic embryogenesis. Caulogenesis is well reported in African violet leaf segments (KuKulcznka and Suszyńka 1972; Redway 1990; Lo 1997; Cassells and Plunkett 1984; Cassels et al. 1986; Start and Cumming 1976; Jain 1993), petioles (Bilkey et al. 1978; Harney and Knop 1979) and floral parts (Molgaard et al. 1991; Vasquez and Short 1978; Weatherhead et al. 1982). Somatic embryogenesis has the benefit of having both a shoot apical meristem and a root apical meristem present at the

same time, therefore, eliminating the need for a rooting step in the production of new plants. Somatic embryogenesis has been reported to occur when exposing African violet explants to the plant growth regulator thidiazuron (TDZ) (Milthia et al. 2003). Shoots or somatic embryos of African violets may be planted and rooted to grow into whole, new plants when planted on a rooting medium. However, using a rooting medium means that there is more time and money producing new plants.

Synthetic seeds, or synseeds, may be used to alleviate this extra expenditure of time and money. A synseed allows for the conversion of meristematic tissue to whole plants under either *in vitro* or *in vivo* conditions (Capuano et al. 1998). Synseeds are analogous to seeds produced by plants because they have a protective covering that allows growth and development of plant tissue. Synseeds are made through the use of an encapsulation agent, commonly sodium alginate (Saiprasad 2001), and a complexing solution, commonly calcium chloride. Most natural seeds contain endosperm that the embryo utilizes to support its development, and likewise a synseed may contain nutrients that can support the growth of the encapsulated meristematic tissue. Synseeds may also contain fungicides or antimicrobial agents to inhibit fungal and/or bacterial growth, therefore increasing survival of the plant tissue (Bapat and Rao 1990). This use of synseeds allows direct planting of propagules in a greenhouse or in the field (Reddy et al. 2005).

This research investigated the effectiveness of TDZ on the production of shoots and/or somatic embryos of *S. rupicola* leaf and petiole tissue in culture. The effectiveness of the TDZ was determined via count data for the different concentrations of TDZ evaluated; 0.5µM, 1.0µM, 1.5µM, 2.0µM, 5.0µM, and 8.0µM. Also included were two

different sterile media types: an induction medium followed by a growth medium and an induction medium only. The viability of the shoots and/or somatic embryos were evaluated by isolating the structure and planting on sterile Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) with half strength sugar *in vitro*, as well as planting synseeds under both *in vitro* and *ex vitro*. Different synseed and media treatments were evaluated in the synseed trials. The synseed treatments included: 2% or 3% sodium alginate followed by a test of plant preservative mixture (PPM^{TM}) concentration; 0.0ml/L, 0.25ml/L, 0.5ml/L, 1.0ml/L, and 5.0ml/L. The different media used for the synseeds included sterile MS basal medium (Murashige and Skoog 1962) with half strength sugar, sterile vermiculite wet with half strength MS macro nutrients (Murashige and Skoog 1962), non-sterile vermiculite wet with half strength MS macro nutrients (Murashige and Skoog 1962), and non-sterile soil (5 parts Promix B, 1 part perlite, and 1 part vermiculite). Information obtained from this investigation could lead to an improved system for mass production of African violets, specifically *S. rupicola*, which may be beneficial in the conservation of the species *in situ* and *ex situ*.

Literature Review

African violet ecology

Habitat

Saintpaulia rupicola B.L. Burtt is a critically endangered species of African violet in the Gesneriaceae family of plants. African violets possess a specialized set of environmental needs that limit their distribution to small areas. Most species live on steep, rocky surfaces in dense shade where few other plants can thrive (Johansson 1978). *S. rupicola* lives on limestone outcrops that are covered in shrubs (Johansson 1978). *S. rupicola* is found only in two isolated populations of no more than 100 individuals in the Cha Simba and Mwarakaya regions of Kenya (Eastwood et al. 1998). These populations are under threat primarily because of human activity in the region. The activity includes mainly encroachment and logging that reduces the available habitat area (Eastwood et al. 1998). Tree removal lessens the shade that the African violets use as protection from exposure to the harsh rays of the sun, that dry out the plants.

Reproductive biology

African violets are limited by an inefficient reproductive ecology. The reproduction is limited by population fragmentation which reduces pollinator service (Kolehamainen and Mutikainen 2006). African violets regularly abort their flowers and fruits for unknown reasons. Commonly, fertilized flowers do not develop into fruit in the wild (Kolehamainen and Murikainen 2006), perhaps because the plant devotes more resources to other parts of the plant. Regular abortion of flowers and fruits, especially those from fertilized flowers, and limited pollinator service, greatly decreases the

regeneration of African violets in the wild. This limited regeneration makes the use of alternative propagation methods, such as *in vitro* propagation, necessary.

Many botanical gardens have tissue culture laboratories specifically for plants that are difficult to propagate through traditional horticultural methods (Fay 1992). Using *in vitro* methods is likely to continue to increase as more plant species become endangered and get closer to extinction (Sarsan et al. 2006). Botanical gardens in developing countries are establishing *in vitro* facilities for the recovery and conservation of more threatened plants (Sarsan et al. 2006). Thus, *in vitro* methods are on the rise and require investigation in more plants.

In vitro propagation

A brief history

In vitro propagation is based on the principles established by cell theory, specifically the principle of totipotency developed by Schleiden (1838) and Schwann (1839). Totipotency is the ability of a single cell to produce new cells that grow and develop into an entire organism. This concept of totipotency inspired the German botanist Gottlieb Haberlandt to attempt to obtain the first experimental evidence of totipotency in plants. Haberlandt attempted this by culturing plant cells in nutrient solutions to possibly grow whole plants (1902). He failed in this endeavor because of his choice of explant type and limited nutrients in Knop's (1865) salt solution that he used for culturing cells. The explant types that he chose were fully differentiated cells from leaves of *Lamium pupureum* L., petioles of *Eichhorinia crassipes* (Mart.) Solms, glandular hairs of *Pulmonaria* and *Urtica*, and stamen hairs of *Tradescantia*. The cells he isolated grew in size but did not divide because they were fully differentiated cells. Even though he failed

in demonstrating tissue culture, he still became known at the Father of Plant Cell and Tissue Culture. Haberlandt also predicted the possibility of successfully culturing artificial or somatic embryos from plant tissue.

Walter Kotte, a student of Haberlandt, was able to grow root meristems from pea and corn in a variety of nutrient solutions (1922). Later, Philip Rodney White was the first person to obtain the indefinite growth of cultured plants. White was able to maintain excised root tips from tomato in culture media for indefinite periods of time (1934), using Knop's (1865) mineral solution. This growth medium is still minimal, however, root tips make a much better explant because there is an apical meristem at the root tip that is already actively performing cell division. Since apical meristems are areas of active cell division there are no fully differentiated cells. Thus, the cells are capable of experiencing further growth and development.

Other scientist began to see the same results as White with the growth of root tips, namely carrot, and tobacco stem tissues (Gautheret 1934, 1939, 1985; Nobecourt 1939). This came about with the use of more nutrient rich media, the new discovery of the phytohormone indole-3-acetic acid (IAA), better choice of explant material, and the use of more aseptic techniques. After this, White decided that there needed to be a new formulation for plant tissue culture that was more nutrient rich. White created what is referred to as White's medium (White, 1943, 1963). This medium was based on the formulation for algal medium made by Uspenskaia (1925) and the microelements found in Trelease and Trelease (1933).

The incorporation of phytohormones, namely auxin, to growth media increased the growth of tissues from dicotyledonous plants, but this was not the case for

monocotyledonous plants. This was solved by Morel and Wetmore (1951) with the use of work from Caplin and Steward. Caplin and Steward (1948) were able to grow cultures of carrot with the use of coconut milk. Morel and Wetmore (1951) then attempted using coconut milk in cultures of monocotyledons and were able to attain growth. The growth promoting factor that is in coconut milk was now the question of interest.

The growth promoting factor in coconut milk was discovered indirectly by Skoog and Tsiu (1948). This discovery was that adenine enhances cell proliferation and budding when included in the culture media. Miller, Skoog, Okumura, Von Saltza, and Strong later isolated kinetin and, thus, discovered the first cytokinin (1955). After this it was found that the cytokinin zeatin is in coconut milk. Upon seeing this, a medium was proposed by Murashige and Skoog (1962) that incorporated auxins, cyokinins, and new mineral solutions that allowed growth of most plant tissues. That medium formulation is now referred to as MS medium and is widely used in many tissue culture laboratories.

Plant growth regulators

A brief history

Phytohormones were first reported by Charles Darwin (1880) when he described the growth of seedlings toward light. However, he didn't know that a phytohormone was causing this action to occur. Boysen-Jensen (1910) elaborated on Darwin's work and pointed to the possibility of a "material substance" being responsible for the movement of the shoot tips. Frits Went (1928) then successfully isolated this "material substance" and called it "wuchstoff," translating to hormone. This hormone, or phytohormone, was later identified to be a naturally occurring auxin, indole-3-acetic acid or IAA. IAA was first isolated in a crystalline structure from the urine of pregnant women by Kögl (Kögl et al.

1934; Kögl and Kostermans 1934). It was then isolated from cultures of the fungus *Rhizopus suinus* by Thimann (1935).

Thimann and his students, including James Bonner and Folke Skoog, continued to study auxins and greatly increased the knowledge of their structure and function, and created a wide variety of synthetic auxins, such as 2,4-dichlorophenoxyacetic acid (2,4-D), that are commonly used in plant tissue culture (Vasil 2008). These auxins have a large degree of control over cell expansion, cell wall acidification, initiation of cell division, and organization of meristems that can promote the growth of unorganized tissue (callus), specific organs (usually roots), and promote vascular tissue differentiation (Gaspar et al. 1996). These functions make the inclusion of auxin in plant culture media paramount when attempting to grow new tissues.

Another class of phytohormone that is of importance in the culture of plants is cytokinins. The first cytokinin discovered was kinetin (Miller et al. 1955). Cytokinins function to stimulate cell division, release of lateral bud dormancy, and the induction of adventitious buds (Gaspar et al. 1996). Cytokinins work together with auxins to regulate cell division by each influencing different parts of the cell cycle (Gaspar et al. 1996). Thus, it is important to control the ratio of auxin to cytokinin in a culture medium to effectively induce growth in the explant (Skoog and Miller 1957).

Thidiazuron in culture

One cytokinin of note is thidiazuron (N-phenyl-N'-1,2,3-thidiazol-5-ylurea), referred to as TDZ. This chemical functions like a phytohormone, however, it is synthetic and, therefore, a plant growth regulator. TDZ is used to induce plant tissues to respond in

a variety of ways including callus and somatic embryo formation (Murthy et al. 1998). Though TDZ is a cytokinin, it possesses the unique ability to function as both a cytokinin and an auxin when incorporated in plant growth media (Murthy et al. 1998). This ability allows TDZ to be used as a substitute for both the auxin and cytokinin requirements for somatic embryogenesis (Saxena et al. 1992; Visser et al. 1992; Gill et al. 1993). TDZ has been claimed to induce somatic embryogenesis in various plants in the Generiaceae; such as *Santpaulia ionantha* Wendl. (Ghorbanzade and Ahmadabadi 2014; Mithila et al. 2003), *Aeschynanthus radicans* Jack (Cui et al. 2009), *Primulina tabacum* Hanson (Yang et al. 2012; Ma et al. 2010), and *Chirita longgangensis* W.T. Wang (Tang et al. 2007).

Somatic embryogenesis

A brief history

Somatic embryogenesis is the development of embryos that are not the products of gametic fusion. The development of somatic embryos is a natural phenomenon in some plant species. It has been noted that plants in the Rutaceae regularly develop somatic embryos from the nucellar tissue in addition to zygotic embryos (Esan 1973). Pine trees are also known to form somatic embryos as a result of polyembryony in their seeds.

Haberlandt did not know about natural somatic embryogenesis, however, he was still the first to envision the possibility of this in culture because of his work in tissue culture (1902). Somatic embryogenesis was first achieved by Frederick C. Steward (1958) in cell suspension cultures and Jakob Reinert (1959) in callus cultures of carrot. The somatic embryos should resemble zygotic embryos with the necessary root, shoot,

and cotyledonary organs (Ammirato 1990). Somatic embryos should also be able to grow into a new whole plant.

The early work with somatic embryogenesis yielded two important discoveries. One, is that an auxin or an auxin-like substance is necessary for embryo initiation (Halperin and Wetherell 1965; Halperin 1966). Two, a source of reduce nitrogen is important for the initiation (Halperin and Wetherel 1965; Halperin 1966) and maturation of somatic embryos. The source of reduced nitrogen can be found in MS medium in the form of ammonium nitrate (NH_4NO_3) and potassium nitrate (KNO_3).

The induction of somatic embryos *in vitro* is accomplished by altering the developmental pathways of plant cells through the introduction of stress factors. If the stress level exceeds the tolerance of the cells the tissue dies, but if the stress is at lower levels the metabolism of the cells is enhanced and adaptive mechanisms begin to function (Lichtenthaler 1998). When plant tissue is exposed to *in vitro* growth conditions the plant experiences significant stress factors; such as removal from the parent tissue and being placed onto medium with non-physiological concentrations of growth regulators, salts, and organic components (Feher et al. 2003). The stress that results from the introduction of the factors stimulates the undifferentiated cells of the explant to begin the process of differentiation. Along with promoting differentiation of cells the stressors can promote the cells to differentiate into somatic embryos (Feher et al. 2003).

In vitro propagation of African violets

A brief history

Kukulczanka and Suszyńska (1972) were the first to culture leaves of *Saintpaulia ionantha in vitro*. This was done through the addition of either kinetin, naphthaleneacetic acid (NAA), or IAA, or various combinations of the three (Kukulcznka and Suszyńka 1972). After this first attempt, many others have been able to achieve tissue culture in leaf segments (Redway 1990; Lo 1997; Casslls and Plunkett 1984; Cassels et al. 1986; Start and Cumming 1976; Jain 1993; Smith 2012), petioles (Bilkey et al. 1978; Harney and Knop 1979), and floral parts (Molgaard et al. 1991; Vasquez and Short 1978; Weatherhead et al. 1982). The final product form these explants was adventitious shoots. These shoots were obtained from the use of phytohormones and plant growth regulators such as: NAA, 2,4-D, IAA, and benzyladenine or benzylaminopurine (BA or BAP) in various combinations and concentrations.

Somatic embryogenesis in African violets

The plant growth regulator TDZ has been shown to induce high-frequency somatic embryogenesis in many other plant species alone or in combination with other plant growth regulators as well (Murthy et al. 1998). Many plants in the Gesneriaceae, including *Saintpaulia ionantha* (Mithila et al. 2003; Ghorbanzade and Ahmadabadi 2014), *Aeschynanthus radicans* (Cui et al. 2009), *Primulina tabacum* (Ma et al. 2010; Yang et al. 2012), and *Chirita longgangensis* (Tang et al. 2007), have been reported to produce somatic embryos under the influence of the plant growth regulator TDZ. This is due, in part, to the cytokinin and auxin like properties of TDZ that allow it to be used for somatic embryogenesis in many species (Murthy et al. 1998). Mithila et al. (2003) used

TDZ and claimed that at lower concentrations (2.5 μM) adventitious shoots were obtained and at higher concentrations (5-10 μM) somatic embryos were obtained.

Synthetic seeds

Synthetic seeds, or synseeds, are artificial seeds that allow the conversion of meristematic tissue into whole plants under either *in vitro* or *in vivo* conditions (Capuano et al. 1998). The meristematic tissue could be somatic embryos, shoot tips, axillary buds, or any number of other tissue that contains a meristem. Synseeds are analogous to seeds produced by plants because they have a protective covering that allows for the growth and development of the plant tissue. The idea of synseeds was first envisioned by Toshio Murashige (1977) as a method for recovering whole plants from the prossess of tissue culture. However, it was Keith Redenbaugh (1984) that first made functioning synseeds from alfalfa and celery.

Explants

Many different explant types have been used for the production of synseeds. Somatic embryos are frequently used because they have the ability to produce roots and shoots at the same time due to having both a shoot and root apical meristem present (Kitto and Janick 1982, 1985; Kim and Janick 1987, 1989, 1990; Janick et al. 1989; Redenbaugh et al. 1984; Redenbaugh et al. 1991; Gray et al. 1991; Redenbaugh 1993; McKersie and Bowley 1993). After much work with somatic embryos, other vegetative material was used for the production of synseeds. Among these vegetative materials were shoot tips of *Morus indica* (Bapat et al. 1987), axillary buds of *Camellia sinensis* (Mondal et al. 2002), calli of *Allium sativum* (Kim and Park 2002), bulblets of *A. sativum*

(Bekheet, 2006), cell aggregates derived from horseradish roots (Repunte et al. 1995), protocorm like bodies of *Geodorum densiflorum* (Datta et al. 1999), and microtubers, rhizomes, and corms (Bapat and Minal 2005).

Encapsulation agents

The main challenge for the production of synseeds was the composition of the encapsulation agent. Eight chemical compounds were tested for the production of the synthetic seed coats. These chemical compounds were polyox, polyco 2133, agar, agarose, alginate, carboxy methylcellulose, carrageenan, guar gum, gelrite, tragacanth gum, sodium pectate ethyl cellulose, nitrocellulose, and polyacrylamide (Ara et al. 2000; Saiprasad 2001; Lambardi et al. 2006). Polyox, a water soluble resin, was proposed as the most suitable agent for the encapsulation of somatic embryos (Kitto and Janick 1982, 1985). However, Redenbaugh et al. (1984, 1986, and 1987) tested and proposed sodium alginate as the most suitable encapsulation agent. Sodium alginate became the most accepted hydro-gel for the production of synthetic seeds because of its low toxicity, low cost, quick gelation, and plant compatibility (Saiprasad 2001).

Encapsulation method

The hydrogel encapsulation method, introduced by Redenbaugh et al. (1987), is the most accepted method for synseed production. In this method, sodium alginate is the hydro-gel used in different concentrations (2-5%). The sodium alginate solution can be prepared by dissolving in a calcium free solution. Explants are then added and mixed into the sodium alginate solution. Then, using a pipette, explants and sodium alginate are picked up to be dropped into a calcium chloride solution. This causes an ion exchange

reaction to take place, where sodium ions are replaced with calcium ions to cross link the alginate molecules together to form calcium alginate beads or seeds. The size of the seeds is entirely dependent on the inner diameter of the pipette. The firmness of the seeds is dependent on the concentration of sodium alginate and calcium chloride solutions, and the complexing time (i.e. time in the calcium chloride solution).

Matrix Additives

Most natural plant seeds contain not only an embryo but also endosperm as a source of nutrients for the developing embryo. It is thought that synseeds should also contain matrix additives to nurture the plant tissue contained within. The matrix additives can contain nutrients or plant growth regulators that can increase the viability and conversion of the seeds. This impacts the quality of the seeds due to temporal, qualitative, and quantitative supply of growth regulators and nutrients (Senaratna 1992). Synseeds may also contain substances such as fungicides and antibiotics that may serve to help protect the plant material when it is beginning to grow.

Advantages of synseeds

There are many advantages associated with using synseeds to propagate plants from *in vitro* methods. The use of synseeds allows direct planting of propagules in a greenhouse or the field (Reddy et al. 2005). Direct planting allows intermediate steps such as acclimatization to be skipped as the plant develops in a more natural manner. Additives also can be included to improve viability of synseeds. Addition of fungicides to the synseed matrix was shown to prevent contamination and increased survival of mulberry buds when they were planted in soil (Bapt and Rao 1990). Addition of activated

charcoal was shown to increase emergence of banana shoot tips from synseeds (Hassanein et al. 2011).

Materials and Methods

Plant Material

Axenic *Saintpaulia rupicola* cultures were originally obtained from Dr. Valerie Pence of the Cincinnati Zoo and Botanical Garden Center for Conservation and Research of Endangered Wildlife. These plants have been kept and cultivated in the same greenhouse at James Madison University for many years. The greenhouse was kept at an average of 25 C with a 16:8 hour light cycle. The plants used in this thesis were placed in tissue culture approximately two years before the start of this thesis and are thus the same relative age and size. These plants were propagated from previously tissue cultured material, therefore limiting the amount of genetic variation between individuals.

Caulogenesis

The medium used for the establishment of tissue cultures was Murshige and Skoog (MS) basal medium (1962) modified by adding thidiazuron (TDZ) in one of six concentrations: 0.5 μM, 1.0 μM, 1.5 μM, 2.0 μM, 5.0 μM, and 8.0 μM (Table 1). Prior to the 0.5 μM, 1.0 μM, and 1.5 μM TDZ treatments, only the 2.0 μM, 5.0 μM, and 8.0 μM TDZ treatments had been completed. These prior experiments included another medium treatment of either an induction medium only or an induction medium for 10 days followed by a basal medium for growth. The pH of the medium was adjusted to 5.75 by adding 1 N HCl and/or 1 N NaOH as necessary prior to the addition of the agar. Agar was then added at 8g/L, and after dissolving, the medium was dispensed in 25 ml aliquots into 25 X 150 mm test tubes. The tubes were then capped with Kaputs and autoclaved for 15 min at 121 C and 15 psi.

Table 1: Components of Murashige and Skoog basal medium (1962) modified with the addition of TDZ in the range used in this thesis.

	Components	**mg/L**
Macronutrients	NH_4NO_3	1,650
	$CaCl \cdot 2H_2O$	440
	KNO_3	1,900
	$MgSO_4 \cdot 7H_2O$	370
	KH_2PO_4	170
Micronutrients	$Na_2MoO_4 \cdot 2H_2O$	1.25
	$CuSO_4 \cdot 5H_2O$	0.125
	$CoCl_2 \cdot 6H_2O$	0.125
	$MnSO_4 \cdot H_2O$	16.9
	$ZnSO_4 \cdot 7H_2O$	10.6
	H_3BO_3	6.2
	KI	0.83
Iron	$Na_2EDTA \cdot 2H_2O$	37.2
	$FeSO_4 \cdot 7H_2O$	27.8
Vitamins	Nicotinic acid	0.5
	Pyridoxine·HCl	0.5
	Thiamine·HCl	0.4
Growth Regulator	TDZ	0.110-1.762
Carbohydrates	*Myo*-inositol	100
	Sucrose	30,000
Organic supplements	Adenine sulfate	80
Agar	Bacto agar	8,000

Leaf and petiole tissues were taken from a clonal population of 25 *S. rupicola* plants to be used for the establishment of tissue cultures. Both tissues were used in the first batch of experiments, and later batches used only leaf tissue. Tissue being used was of the same approximate age, size, and developmental stage. After removal, the tissue was gently swirled in a solution of approximately 500 ml distilled water with 2 to 3 drops of Tween 20, a surfactant, to be rinsed. The tissue was then moved into a sterile laminar flow hood to be placed into a sterile beaker containing a 10% Clorox bleach (0.825% sodium hypochlorite) solution for surface sterilization. The sterilized tissue was left in a beaker with approximately 100ml of sterile distilled water to prevent desiccation as only

one piece of tissue may be prepared for culture at a time. Each petiole was cut into sections about 2mm long. Each leaf had the midrib and margin excised, and was sectioned into 8-10 explants approximately 2 cm wide and 3 cm tall (Figure 1). Each explant was planted on the appropriate medium with a vein in contact with the surface whenever possible. The tubes with explants were then sealed with Parafilm and put into a test tube rack. The adjacent slots to each sample on the rack were left empty to allow optimal amounts of light to reach each explant. The tubes were then stored in a growth chamber with a temperature of 24.5 C, photoperiod of 16:8, and an average photon flux of 90 μmol/m^2/s. The tubes were observed weekly for any change, deterioration or growth. After 12 weeks, a final observation was made, and all the structures present were counted. These structures for which cultures were observed included somatic embryos, shoots, callus, and roots. Throughout, the cultures were observed weekly for growth and contamination. Any cultures found to be contaminated were removed from the study as they were in violation of the parameters of the experiment.

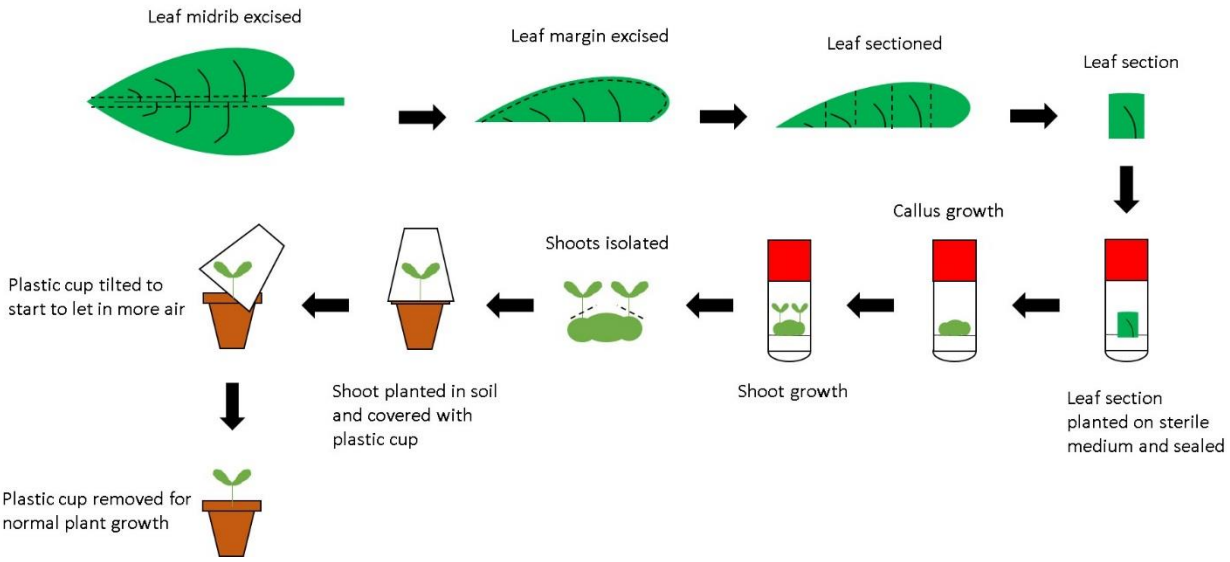

Figure 1: Flow chart of *S. rupicola* propagation from leaf tissue.

Upon completion of growth and observation, callus and shoots were removed from the cultures under sterile conditions in a laminar flow hood. The removed callus and shoots were planted on MS basal medium (1962) modified with half strength sucrose (15,000 mg/L) in 25 X 150 mm test tubes. These cultures were left for approximately one month and checked once a week for growth. Isolated shoots were observed for the continued production of new leaves. Isolated callus was observed for an increase in size, as well as differentiation, the production of shoots, roots, and/or somatic embryos. After the shoots grew and developed roots, they were removed from their test tubes and planted in 7.62 cm plastic pots with soil based medium containing 5-parts ProMix B, 1-part perlite, and 1-part vermiculite. The pots were covered with inverted small clear plastic cups to begin the acclimatization process. After about a week, the cups were tilted slightly to begin to decrease the humidity. The following week, the cups were removed from the pots to complete the acclimatization.

Synthetic seeds

The use of synthetic seeds, or synseeds, as a planting method was evaluated. Shoots for encapsulation were obtained from fresh *S. rupicola* cultures during their final observation. The complexing agent was 100 mM $CaCl_2$, the complexing time was 20 min, and the rinse time was 10 min (Figure 2). The manipulated variable was the composition of the synseed matrix.

The first encapsulation method tested was based on the percentage of sodium alginate, 2% (*w/v*) or 3% (*w/v*). The two treatments were used to create 80 synseeds each. These synseeds were planted on MS basal medium (1962) modified by using half

strength sucrose. These plants were observed periodically for emergence with a final observation being made after approximately two weeks. The plant was recorded as emerged when two whole leaves grew out of the synseed and into the air.

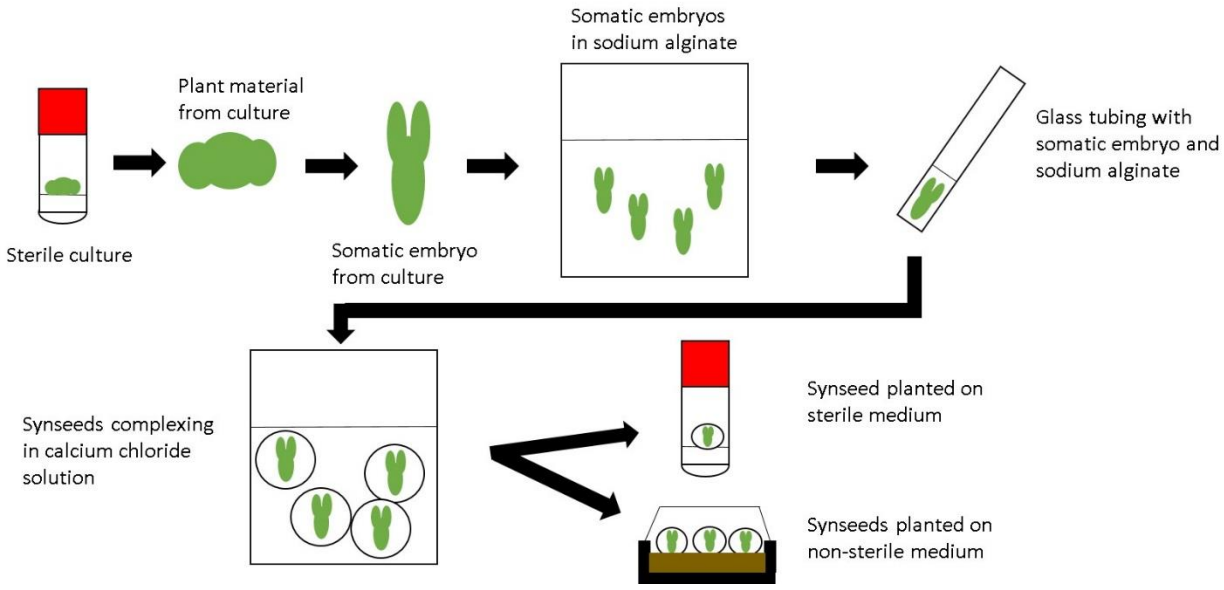

Figure 2: Flow chart of *S. rupicola* synseeds creation.

Based on the data from the previous experiment, 2% (*w/v*) sodium alginate was used for the creation of synseeds modified with other matrix components because this treatment had a higher rate of emergence. Plant preservative mixture^{TM} (PPM^{TM}) was then included in the synseed matrix in one of five concentrations: 0.0 ml/L, 0.25 ml/L, 0.5 ml/L, 1.0 ml/L, and 5.0 ml/L. These seeds were first planted on MS basal medium (1962) modified with half strength sucrose to evaluate the potential phytotoxicity of PPM^{TM}.

Those data were then used to determine which synseed treatment would be used for trials on different media. Due to preliminary results the 5.0 ml/L PPM^{TM} synseeds

along with 0.0 ml/L PPM^{TM} synseeds were used for the different media trials, because there was no significant difference among the PPM^{TM} treatments the highest concentration was selected. The first medium tested was vermiculite that filled approximately half of the 25 X 150 mm test tubes, was wetted with half-strength MS macro-nutrients (1962), capped with Kaputs, and autoclaved at 121 C and 15 psi for 15 min. Non-sterile media was then used in flats with seed trays that had a total of 48 cells, each measuring 6 cm X 4 cm X 5.5 cm. A total of eight flats were used with two flats containing vermiculite and the other two containing a soil mix consisting of 5-parts ProMix B, 1-part perlite, and 1-part vermiculite. The two vermiculite flats were wetted with half-strength MS macro-nutrients (1962) and the two soil flats were wetted with water. One flat of each treatment were planted with 5.0 ml/L PPM^{TM} synseeds and the others were planted with no PPM^{TM} synseeds for comparison. After planting, a dome was put on top of each flat to retain moisture. Every two days, the dome was removed to spray the medium with de-ionized water (to avoid chlorination in tap water) to maintain consistent moisture. The flats were observed each day for emergence of plants as well as fungi growth on the medium. Cells with observable fungi were immediately emptied and removed from the experiment.

Statistical analysis

All data were analyzed using the program R version 3.4.4. To determine what test to use all datasets were initially analyzed for normality and variance prior. A Wilcox Rank Sum Test was conducted to determine the significance of the differences in growth on the induction and growth media treatment (n = 118) and the induction only medium

treatment (n = 117). A Mood's Median Test was conducted to analyze significance of the differences in number of shoots produced among the induction and growth media treatments (2.0 µM (n = 39), 5.0 µM (n = 39), and 8.0 µM (n = 40) TDZ) and the induction only media treatments (2.0 µM (n = 39), 5.0 µM (n = 39), and 8.0 µM (n = 39) TDZ). A Pairwise Comparison using Mood's Median Test was then used to identify where the significant differences were among the treatments. A Kruskal-Wallis Rank Sum Test was conducted to analyze the significance of the differences in number of shoots produced among the lower concentration TDZ treatments on induction media only (0.5 µM (n = 40), 1.0 µM (n = 40), 1.5 µM (n = 40), and 5.0 µM (n = 39) TDZ). A Dunn Kruskal-Wallis Multiple Comparison was then used to identify any significant differences between the treatments, using the Bonferroni method. A Fisher's Exact Test was conducted on the synseed encapsulation percentage (2% (n = 80) and 3% (n =80)), PPM^{TM} inclusion on sterile medium (0.0 ml/L (n = 60), 0.25 ml/L (n = 59), 0.5 ml/L (n = 59), 1.0 ml/L (n = 59), and 5.0 ml/L (n = 58)), PPM^{TM} inclusion sterile vermiculite (0.0 ml/L (n = 80) and 5.0 ml/L (n = 80) PPM^{TM}), PPM^{TM} inclusion on non-sterile vermiculite (0.0 ml/L (n = 48) and 5.0 ml/L (n = 48) PPM^{TM}), and PPM^{TM} inclusion on non-sterile soil (0.0 ml/L (n = 48) and 5.0 ml/L (n = 48) PPM^{TM}) to determine the significant differences in plant emergence seen within each of the datasets. Pairwise Comparisons using Pairwise Comparison of Proportions was used on the 0.0 ml/L and 5.0 ml/L PPM^{TM} synseed treatments on all of the different media used to determine significant differences in the proportion of plants that grew within each treatment.

Results

Establishment of *in vitro* cultures

Petiole explant cultures did not show any signs of growth, of any sort, in the 12-week observation period (Table 2). Leaf explant cultures produced up to and in some cases over 20 shoot primordia that could be used for rooting on a basal medium or encapsulation (Table 2). All explants that grew produced callus first, from which shoot primordia differentiated. None of the explants produced any observable somatic embryos.

Table 2: Percent of explants that developed any shoot primordia comparing petiole and leaf explants with the media type that was used.

Explant type	**Induction and Growth**	**Induction Only**
Petiole	0% (n = 60)	0% (n = 60)
Leaf	58.47% (n = 118)	82.91% (n = 117)

Leaf explants that were planted only on an induction medium (sustained exposure to plant growth regulator) produced significantly more ($W = 3407.5$, $p = <0.001$) shoots than those planted on an induction medium for 10 days followed by a basal growth medium (no plant growth regulator) (Figure 3).

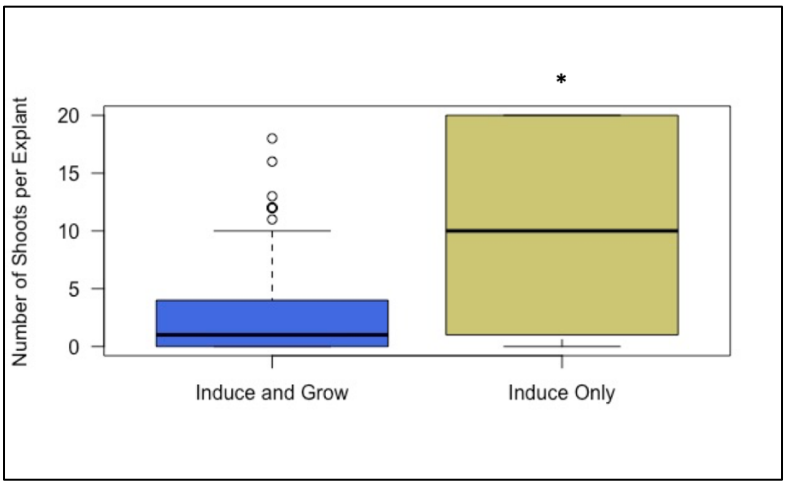

Figure 3: Number of shoot primordia produced from the two different media treatments. Induce and Grow (n = 118) is the induction medium followed by the basal growth medium and Induce Only (n = 117) is the induction medium alone. The * denotes significance from a Wilcox Rank Sum Test with p = <0.001.

Each of the two treatments, induce and grow (IG) and induce only (IO), consisted of three TDZ concentrations, 2.0 μM, 5.0 μM, and 8.0 μM. There were significant differences between the treatments tested (X-squared = 40.153, p = <0.001, Figure 4). The treatment IO 2.0 μM was significantly greater than IG 2.0 μM and IG 8.0 μM (Figure 4, Table 3). The treatment IO 5.0 μM was significantly greater than all of the other treatments except IO 8.0 μM (Figure 4, Table 3).

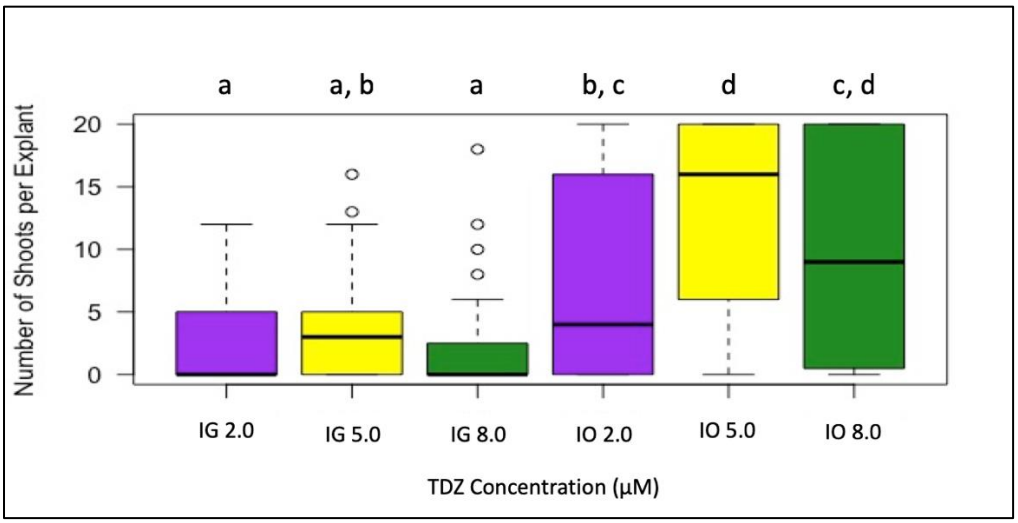

Figure 4: Comparison of the different media types Induce and Grow (IG) and Induce Only (IO) with the three concentrations of TDZ used in each. The letters above the graph indicate significant differences from the Pairwise Comparison using Mood's Median Test.

Table 3: Table of significant p-values from the Pairwise Comparison using Mood's Median Test for the different media types (IG and IO) and the different TDZ treatments. The NS represents p values that are not significant.

	IG 2 μM (n = 39)	**IG 5 μM**	**IG 8 μM**	**IO 2 μM**	**IO 5 μM**
IG 5 μM (n = 39)	NS	-	-	-	-
IG 8 μM (n = 40)	NS	NS	-	-	-
IO 2 μM (n = 39)	0.039	NS	0.024	-	-
IO 5 μM (n = 39)	<0.001	<0.001	<0.001	0.027	-
IO 8 μM (n = 39)	0.020	0.039	0.001	NS	0.390

Upon completion of the first TDZ treatments, new concentrations of 0.5 μM, 1.0 μM, and 1.5 μM TDZ were tested along with another 5.0 μM TDZ trial to compare with the previous treatments because lower concentrations of plant growth regulator may induce somatic embryogenesis. There were no significant differences between the treatments (Kruskal-Wallis chi-squared = 2.1808, p = 0.5357, Figure 5).

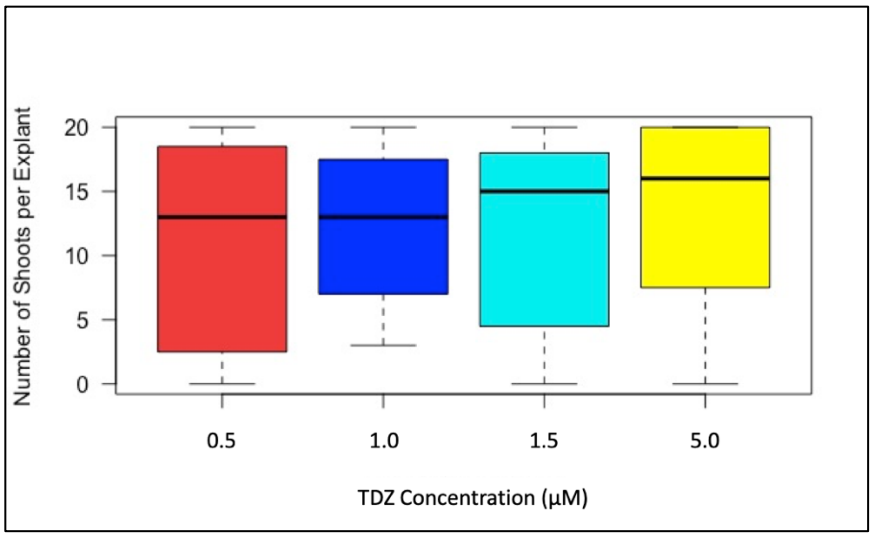

Figure 5: Comparison of induction media only modified with lower concentration of TDZ. Sample sizes from left to right are: n = 40, 40, 40, and 39. There are no significant differences between the treatments (Kruskal-Wallis chi-squared = 2.1808, p = 0.5357).

Isolated shoots and callus on basal medium

All of the shoots that were isolated from each of the different TDZ treatments (n = 6/treatment) were able to grow on the MS (1962) basal medium with half-strength sugar. These shoots first grew in size and then began to form roots which grew into the agar and the air (Figure 6a). There was no contamination seen in any of the tubes with isolated shoots. Callus that was isolated from each of the TDZ treatments (n = 6/treatment) was able to grow in size and then shoots developed when grown on the MS (1962) basal medium with half strength sugar (Figure 6b). There was no contamination observed in any of the tubes with isolated callus. All shoots that were removed from the test tube after rooting *in vitro* (n = 32) were able to be acclimatized and grown under greenhouse conditions, in a soil based medium (Figure 7).

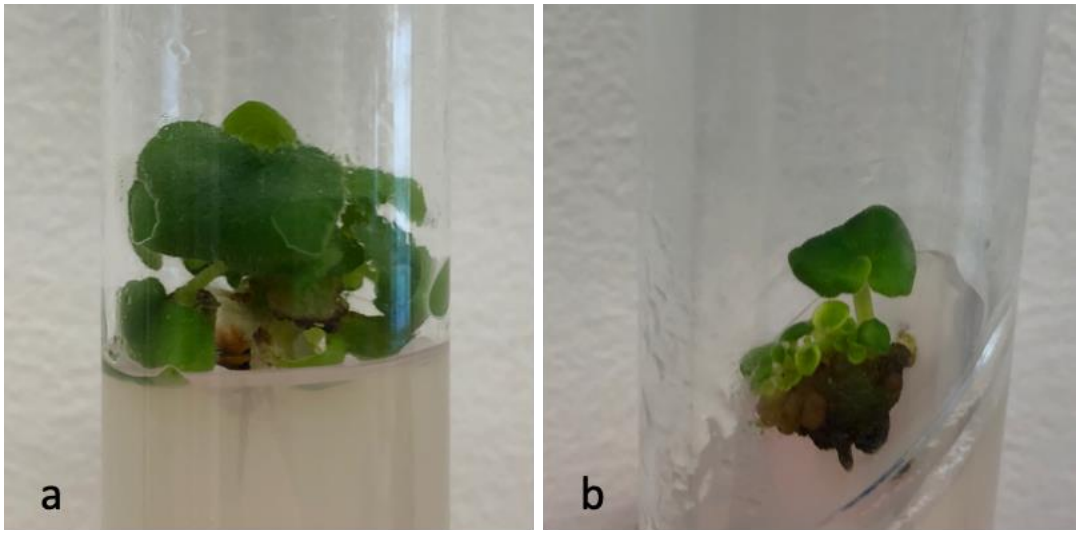

Figure 6: *S. rupicola* (a) shoot with new, larger leaves and roots growing into the agar, and (b) callus with shoots differentiating.

Figure 7: *S. rupicola* (a) planted in soil with plastic cup cover, (b) that are acclimatized and being acclimatized, and (c) that are growing fully acclimatized to the greenhouse.

Synseed encapsulation percentage

Shoot primordia that were isolated from all cultures, as there were no differences in growth after being removed, and were encapsulated in either 2% alginate or 3% alginate to be planted on MS (1962) basal medium with half-strength sucrose. Shoot primordia that were encapsulated in a 2% alginate matrix had significantly more plants

emerge and grow than those encapsulated in a 3% alginate matrix (odds ratio = 0.0896, p = 0.000917, Figure 8).

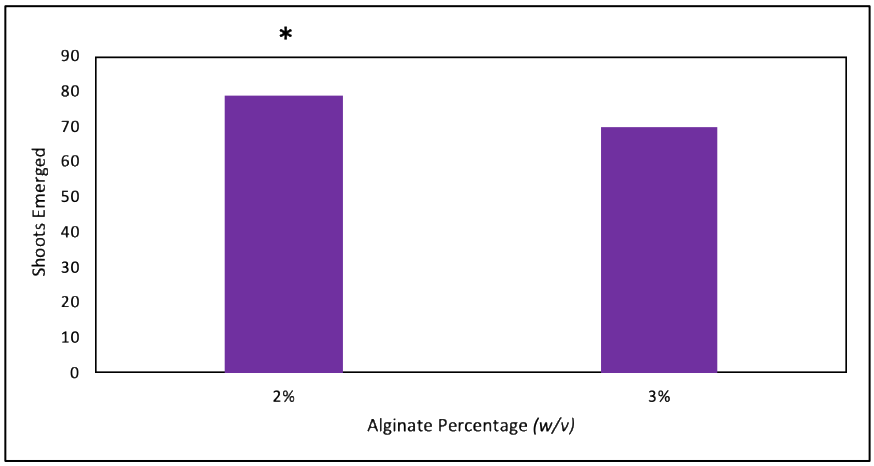

Figure 8: Comparison of alginate percentage treatments, 2% (n = 80) and 3% (n = 80). The * denotes that 2% alginate synseeds had significantly more emerged plants from a Fisher's Exact Test (odds ratio = 0.0896, p = 0.000917).

PPM^{TM} inclusion on sterile media

Two experiments were conducted with PPM^{TM} included in the matrix of the synseed planted on sterile media. The first tested 0.0 ml/L, 0.25 ml/L, 0.5ml/L, 1.0 ml/L, and 5.0 ml/L PPM^{TM} synseeds planted on MS (1962) basal media with half strength sucrose. There was an emergence rate of 82% for all of the PPM^{TM} concentration. There were no significant differences seen in these data from the Fisher's Exact Test that had a p = 0.8642 (Figure 9). The second experiment tested 0.0 ml/L and 5.0 ml/L PPM^{TM} synseeds, since there was no difference between all the PPM^{TM} concentrations evaluated previously, planted on sterile vermiculite that was wet with half-strength MS (1962) macro nutrients. The emergence rate was 52% for both the synseed treatments. There was also no significant difference seen in these data from a Fisher's Exact Test (p = 0.4827, Figure 10).

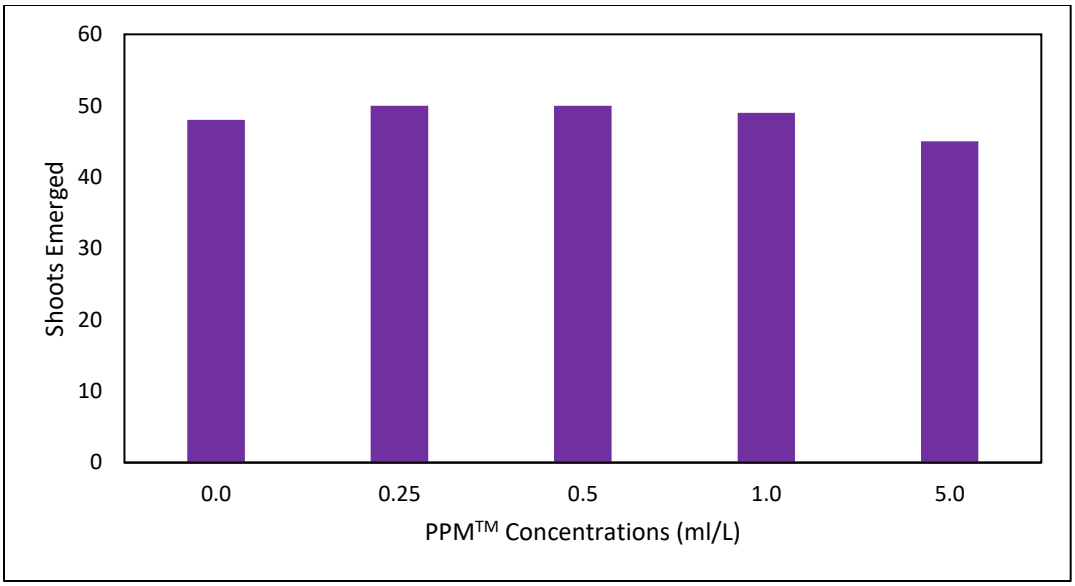

Figure 9: Comparison of the different PPM™ concentration included in the matrix of the synseed, planted on sterile MS (1962) basal medium with half strength sucrose. Sample sizes from left to right are: n = 60, 59, 59, 59, and 58. There were no significant differences between the treatments.

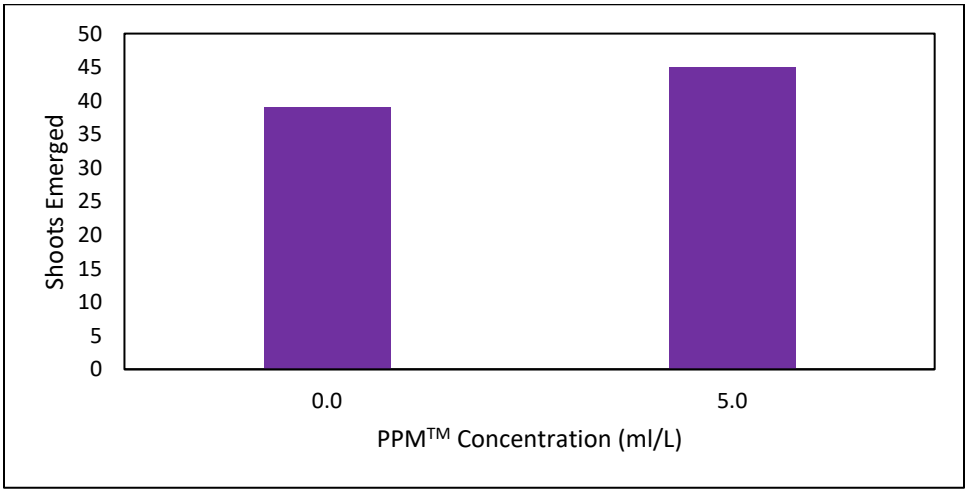

Figure 10: Comparison of 0.0 ml/L (n = 80) and 5.0 ml/L (n = 80) PPM™ concentrations included in the matrix of the synseed, planted on sterile vermiculite wetted with half-strength MS (1962) macro nutrients. There was no significant difference between the treatments.

PPM^{TM} inclusion on non-sterile media

Two experiments were conducted with PPM^{TM} included in the matrix of the synseed planted on non-sterile media. Both of these experiments used the same PPM^{TM} treatments of 0.0 ml/L and 5.0 ml/L PPM^{TM} included in the synseed matrix. The first experiment tested the synseeds planted on non-sterile vermiculite wet with half-strength MS (1962) macro nutrients and the second experiment tested planting on non-sterile soil that was wet with de-ionized water. The non-sterile vermiculite trial had 54% rate of emergence from the synseed in both the synseed treatments. There was no significant difference seen in these data ($p = 0.8378$, Figure 11). The non-sterile soil trial had 13.5% rate of emergence from the synseed in both of the synseed treatments. There was no significant difference seen in these data ($p = 0.833$, Figure 12).

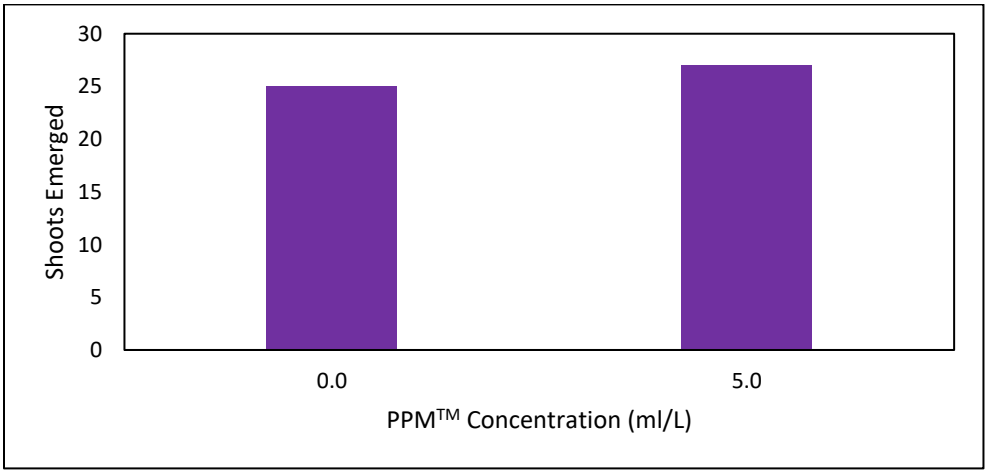

Figure 11: Comparison of 0.0 ml/L (n = 48) and 5.0 ml/L (n = 48) PPM^{TM} concentrations included in the matrix of the synseed, planted on non-sterile vermiculite wetted with half-strength MS (1962) macro nutrients. There was no significant difference between the treatments.

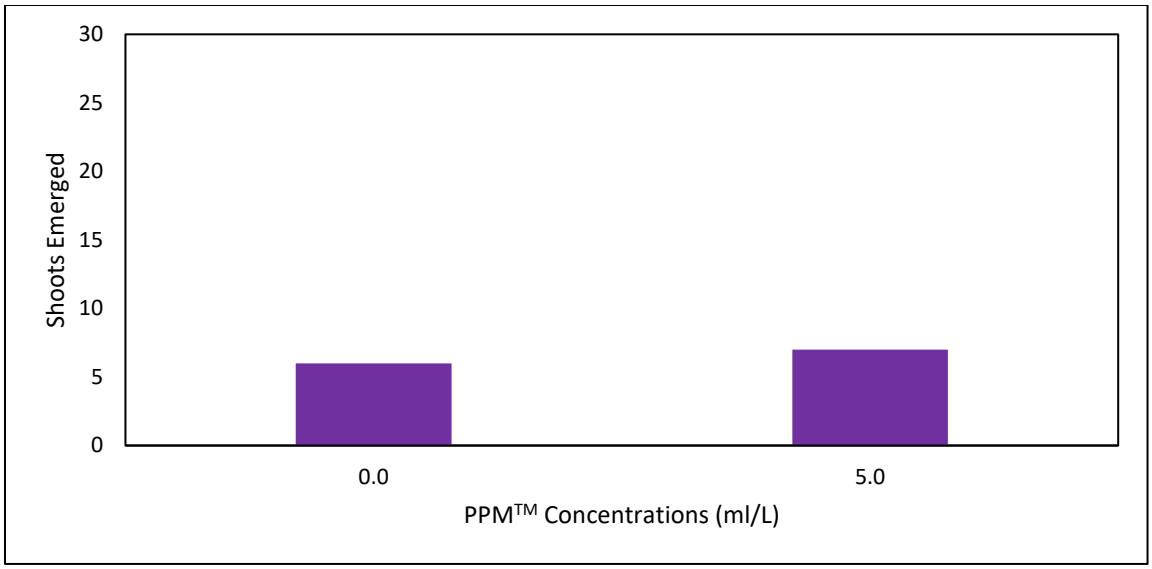

Figure 12: Comparison of 0.0 ml/L (n = 48) and 5.0 ml/l (n = 48) PPM™ concentrations included in the matrix of the synseed, planted on non-sterile soil wetted with de-ionized water. There was no significant difference between the treatments.

PPM™ inclusion among different media:

All of the different combination of PPM™ concentration experiments were combined to compare the different planting media to each other. Pairwise Comparisons using Pairwise Comparison of Proportions was used to determine significant differences among the treatments (Figure 13). Both of the MS (1962) medium with half-strength sugar treatments had significantly more plants grow than the 0.0 ml/L PPM™ synseeds planted on sterile vermiculite wetted with half-strength MS (1962) macro nutrients (Figure 13). Both of the non-sterile soil wetted with deionized water had significantly less plant growth than all the other treatments (Figure 13).

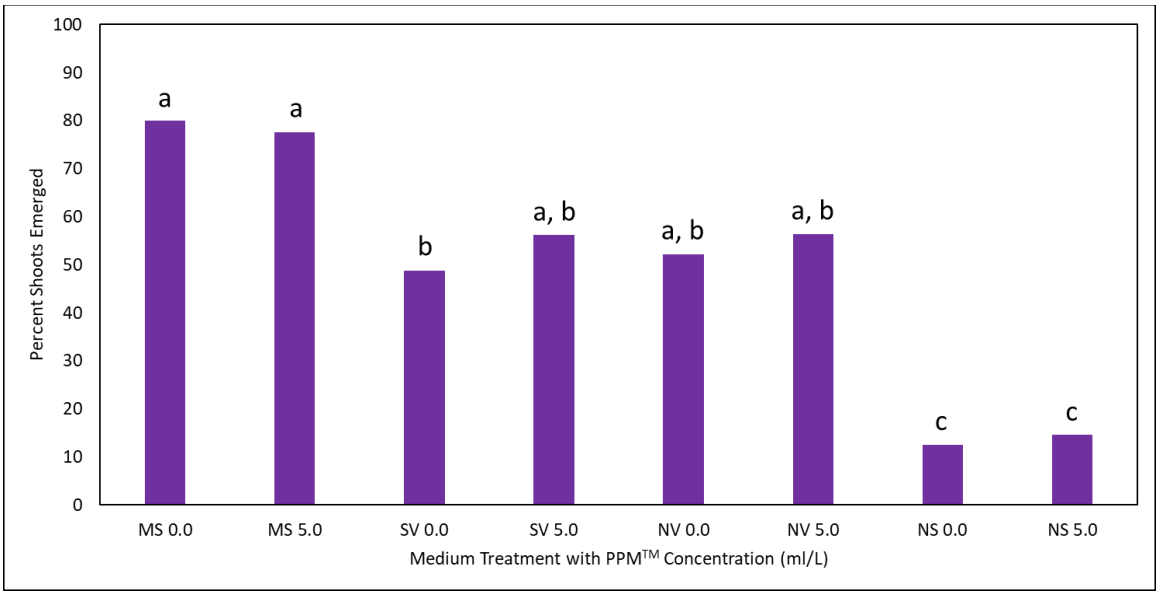

Figure 13: Comparison of the proportion of emerged plants from the different media treatments with 0.0 ml/L and 5.0 ml/L PPM^{TM}. The different treatments are labeled MS for Murashige and Skoog (1962) basal medium, SV for sterile vermiculite, NV for non-sterile vermiculite, and NS for non-sterile soil. The letters above the bars denote significant differences from Pairwise Comparisons using Pairwise Comparison of Proportions.

Discussion

Organogenesis

This investigation was undertaken to help better inform the field on propagation of *S. rupicola in vitro* for both *in situ* and *ex situ* conservation, as this species is critically endangered. There is a great deal of literature that describes the *in vitro* propagation of African violets, however, the vast majority of this literature uses only one species of African violet, *S. ionantha*. The lack of attention paid to *S. rupicola* and the other species of African violet creates a need to test the methods used with *S. ionantha* to ensure that they do indeed function in the entire genus. This sentiment is true for other tissue culture practices as well because, though based in plant physiology and development, there is still a wide range of variability among species and often genotypes (Pence 2013).

This thesis focused specifically on the use of thidiazuron, TDZ, to induce organogenesis in tissues of *S. rupicola*. The concentrations of TDZ used were selected primarily based on the work done by Milthia et al. (2003). This prior work reported that somatic embryogenesis was achieved in *S. ionantha* when utilizing higher concentrations of TDZ in the medium (Milthia et al. 2003). However, the results of this thesis are interpreted differently. A similar number of organs were obtained per explant, but these organs were not seen to be somatic embryos. Structures similar to those described by Milthia et al. (2003) to be somatic embryos were observed and were determined to be abnormal shoots. The use of such high concentrations of TDZ (> 1.5 µM) seems to have interfered with the normal growth of shoots on the explants of *S. rupicola*. Milthia et al. (2003) also lacked histological evidence or further growth of the embryos to later stages of development that would lend support to anyconclusion that they indeed produced

somatic embryos. The reported somatic embryos also had trichomes present (Milthia et al. 2003) and trichomes are not found on the embryos of plants (Evert 2006).

Shoots were only able to be produced by leaf explants as none of the petiole explants exhibited any form of growth in the 12-week observation period (Table 2). This may be due to the high concentrations of TDZ (2 μM, 5 μM, and 8 μM) that were used on the petiole tissue. These concentrations may have been too high for the petiole tissue because there was less petiole tissue per explant than there was leaf tissue. With the increased tissue the leaf explants had, the TDZ being at these high concentrations may have had less of an effect allowing growth of callus and, subsequent adventitious shoots. There were significantly less shoots per explant produced in the induction medium followed by basal growth medium treatment than the induction medium only treatments (Figure 3). This is most likely due to there being too much stress on the tissue from being handled more often and the sudden change in the concentration of plant growth regulator.

The treatments used in both the induction medium and basal growth medium and the induction medium only treatment showed the same general trend of a bell curve from the 2.0 μM TDZ to 8.0 μM TDZ (Figure 4). The 5.0 μM TDZ induce only treatment had significantly more shoots per explant than the 2.0 μM TDZ induce only treatment and no significant difference from the 8.0 μM TDZ induce only treatment (Figure 4). However, upon further experimentation with lower concentrations of TDZ (0.5 μM, 1.0 μM, and 1.5 μM along with a 5 μM) there was no significant differences seen (Figure 10). This observed difference in outcomes is likely due to improvement in the performance of tissue culture techniques. The better results indicate that less TDZ (< 1.5 μM) can be used in the production of shoots to make new plants. This reduces the cost per plant produced,

as TDZ is an expensive plant growth regulator. Though none of these treatments resulted in the production of somatic embryos, the 0.5 μM and 1.0 μM TDZ treatments were the only ones to produce adventitious shoots and roots independent of each other; suggesting that lower TDZ concentrations may be needed for the induction of somatic embryogenesis in *S. rupicola*. These concentrations still need to be evaluated in liquid media with a cell suspension of *S. rupicola* because liquid culture is another technique for the induction of somatic embryogenesis (Stewart et al. 1958; Reinert 1959; and von Arnold 2002).

Synseeds

Synseed technology has the potential to be used in the production of plants under both *in vitro* and *ex vitro* conditions. The use of these synseeds *ex vitro* is promising for the conservation of plants. Synseeds are small and make the transportation of plants to a potential site in the wild for restoration easier. However, synseeds need to be evaluated under greenhouse conditions to be sure that the synseeds are able to be used in the field to help with the conservation of *S. rupicola* and potentially other species. This current investigation evaluates the use of synseeds under both *in vitro* and *ex vitro* conditions to begin to inform how synseeds can be used in the production of *S. rupicola* for potential wild planting.

Synseeds were to be made for the purpose of planting somatic embryos, however, since no somatic embryos were produced in this study shoot tips were used in their place. Shoot tips lack a root axis when planting which causes an increase in the amount of time it takes to achieve an established plant. However, since shoot tips of *S. rupicola* are capable of emerging from synseeds and subsequently rooting this is a method that can be

used for the production of new usable plants. Synseeds were first made with 2% and 3% *(w/v)* sodium alginate complexed in 100 mM $CaCl_2$ for 20 min to determine which concentration might be more effective in the emergence of *S. rupicola*. The 2% sodium alginate allowed emergence of significantly more plants than the 3% treatment did. This was from a difference of only nine more plants emerging in the 2% treatment, though this is a trend that should be investigated further with a larger sample size. However, there is economic significance given the cost of alginate that makes using 2% sodium alginate advantageous. Since the 2% treatment did have more plant emergence it was the concentration that was used for the remainder of the synseed experiments, because it makes a softer synseed matrix that is easier for plants to emerge from.

Plant preservative mixture (PPM^{TM}) was then included as an anti-microbial agent in the synseed matrix in various concentrations (0.0 ml/L, 0.25 ml/L, 0.5 ml/L, 1.0 ml/L, and 5.0 ml/L). These concentrations were selected based on work done with other herbaceous dicot species (Rihan et al. 2012). PPM^{TM} needs to be evaluated for phytotoxicity in each species it is used on because there is a lack of information on the concentrations that may be lethal to the plant tissue (Rihan et al. 2012). PPM^{TM} has also not been included in a synseed matrix but in the medium on which the seeds are being planted. Including the PPM^{TM} in the synseed may prove useful in the prevention of microbial attack on the synseed before and during plant emergence. This strategy may prevent attack while not removing all microbes from the soil, as some microbes may be beneficial to an emerged plant with roots.

There was no significant difference in the emergence between any of the PPM^{TM} concentrations (0.0 ml/L, 0.25 ml/L, 0.5 ml/L, 1.0 ml/L, and 5.0 ml/L) when planted on a

sterile basal medium (Figure 9). This indicates that the encapsulated shoots can tolerate PPM^{TM} concentration at least up to 5.0 ml/L. This allowed use of 0.0 ml/L and 5.0 ml/L PPM^{TM} treatments for further evaluation of the impact of PPM^{TM} concentration in synseeds. This was done with vermiculite wet with half-strength MS macro-nutrients (1962) under sterile conditions to begin to evaluate the differences in plant growth that are seen when the synseeds are planted on different media. There was no significant difference seen in this dataset (Figure 10) allowing further evaluation of these treatments in non-sterile conditions because planting in non-sterile conditions is a more accessible method to conservationists and other growers. Both of these treatments in non-sterile greenhouse conditions had no significant difference between them (Figure 11). However, in both the sterile and the non-sterile vermiculite treatments the 5.0 ml/L PPM^{TM} treatment did have more shoots emerge compared to the no PPM^{TM} (Figures 10 and 11). There was no microbial attack (bacterial or fungal) observed in either of the treatments, but there may have been some unseen microbial interference that resulted in the 5.0 ml/L PPM^{TM} treatment having more shoots emerge. There was no significant difference seen between the 0.0 ml/L and 5.0 ml/L PPM^{TM} treatments on non-sterile soil (Figure 12). The emergence rate for the two soil treatments was very low, 13.5%. This was much lower than the non-sterile vermiculite and may be due to increase in the unknown composition of the soil medium. In the vermiculite treatments the nutrient concentrations were known and the non-sterile vermiculite likely had less microbial activity compared to soil. There was a difference of the 5.0 ml/L treatment having one more plant emerge than the 0.0 ml/L treatment. This is not much of a difference at all and indicates that there is no statistically significant difference in the use of PPM^{TM} in the synseeds planted on non-

sterile soil. The non-sterile soil trials should be repeated and possibly incorporate other concentrations of PPM^{TM} to determine what factor was affecting the soil trials whether it be difference in nutrient composition, texture, or microbial activity.

Agar appears to be the best, though not statically significant, medium to plant the synseeds on for emergence of the plants (Figure 13). However, the use of vermiculite in the greenhouse provides a more cost effective solution to the emergence of usable plants. Within the non-sterile vermiculite treatment there was no significant difference in the emergence of plants from synseeds with and without PPM^{TM} included in the matrix (Figure 11), indicating a further decrease in the cost of producing synseeds for planting in a greenhouse setting.

Conclusion

S. rupicola is a critically endangered plant known only to the Cha Simba and Mwarakaya regions in Kenya, where each population numbers up to only 100 individuals (Eastwood et al. 1998). This plant is critically endangered because of its sensitivity to drought (Johansson 1978), and the encroachment of agriculture (Eastwood et al. 1998) that is removing the canopy cover needed for the plants to receive optimum levels of light. African violets are known to be unsuccessful at reproduction in nature, potentially due to population fragmentation (Kolehamainen and Mutikainen 2006). Limited regeneration means that investigations into alternative propagation techniques, such as *in vitro* propagation, are necessary for the survival of the species. This thesis has presented information on the production of new *S. rupicola* plants through *in vitro* propagation for the purposes of both *ex situ* and *in situ* conservation.

TDZ has been shown to be effective in the production of adventitious shoots that have the capability to grow into new plants usable plants. There is no difference seen in the production of shoots depending on the concentrations evaluated and, therefore, the lower concentrations may be used. Using the lower concentrations of TDZ then provides a more cost effective method than the use of higher concentrations. The results from the TDZ experiments do have the same number of organs as seen in Milthia et al. (2013), however, the structures here were all interpreted to be shoots and not somatic embryos. Therefore, these results refute the claim by Milthia et al. (2013) that the use of TDZ will result in the production of somatic embryos.

The synseed experiments have served to show that synseeds are an effective planting method for the production of new, usable *S. rupicola* plants. It was demonstrated that the shoots can tolerate being planted on a variety of media and various concentrations of PPM^{TM} in the synseeds. However, the use of PPM^{TM} was not seen to have any significant impact on the emergence of the shoots from the synseed. This information allows for the production of cheaper synseeds because PPM^{TM} is not a necessary component in the synseed. However, PPM^{TM} still needs to be tested further to confirm that it is not a requirement. It is important for planting on soil to be reevaluated in future studies. This investigation served to generate a method for planting synseeds in the greenhouse using non-sterile vermiculite wet with half-strength MS macro-nutrients (1962). This is a method that may be used for the mass production of *S. rupicola* plants to be reintroduced to its native range. This method may also be adapted to use in other endangered plant species to assist in their conservation both *in situ* and *ex situ*.

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