EVALUATION OF SCARIFICATION AND STRATIFICATION TECHNIQUES IN BREAKING SEED DORMANCY AND DEVELOPMENT OF A REGENERATION PROTOCOL FOR *ROSA RUBIGINOSA*

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DECLARATION

This thesis is my original work and has not been submitted to any other University for examination.

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DEDICATION

I would like to dedicate this work to my parents Mrs. 'Maitumeleng Mokhobo and Mr. Serame Mokhobo and my brother Letlatsa David Ramabele for their unwavering support throughout the execution of this project.

ABREVIATIONS

AA	Ascorbic acid
ABA	Abscisic acid
AC	Activated charcoal
AT-325C	Azoxy Top 325SC
AWP-70	Antracol WP 70
BAP	6-benzylaminopurine
GA3	Gibberellic acid
GUS	β-*glucuronidase*
IAA	Indole-3-acetic acid
MS	Murashige and Skoog
NAA	Napththalene acetic acid
NaClO	Sodium hypochlorite
RG-MZ	Ridomil Gold MZ
SDW	Sterile distilled water
2,4-D	2,4-dichlorophenoxyacetic acid

ABSTRACT

Rosa rubiginosa is popular in Lesotho where rural communities sell the rosehips to the local companies. However its deep seed dormancy and absence of a regeneration protocol presents a challenge in establishing its plantations for sustainable use. Thus, this study aimed at evaluating scarification and stratification techniques in breaking seed dormancy and also to develop regeneration protocol for *R. rubiginosa*. Hot water treatment in combination with acid scarification plus warm and cold stratification techniques were used though all the treatments failed to break the dormancy. Moreover, dye penetration and imbibition tests proved that hot water treatment did not improve penetration capacity and imbibition properties of the seeds. For direct regeneration using nodal explants shoot induction of 100% was obtained on MS medium treatments with 0.5 $mg^.L^{-1}$AA, 0.5 $g^.L^{-1}$ AC or 2.0 $mg^.L^{-1}$ BAP, beyond these concentrations shoot induction was inhibited. No significant difference observed in shoot induction between the nodal explants cultured in either vertical or horizontal orientation. However, all the shoots failed to root in different rooting trials. Rapid response of callus formation from leaf explants was observed after 4 weeks with highest percentage of 80% on medium with 1.5 $mg^.L^{-1}$ 2,4-D however all attempts for shoot induction from induced callus were not successful. In conclusion, shoot induction protocol was successfully developed however, for a complete regeneration protocol more research is required to induce rooting. Moreover, molecular approaches should be attempted for breaking seed dormancy.

Keywords: *Rosa rubiginosa*, seed dormancy, regeneration,

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CHAPTER ONE

1.0   Background of the Study

Rosa rubiginosa also known as sweet briar is a deciduous plant belonging to *Rosaceae* family and it is characterized by prickly and bristled shoots (Werlemark, 2009). The economic importance of *Rosa* species have been reported since the ancient time (Werlemark, 2009). The rosehips from *R. rubiginosa* are used to make medicine prescribed for treatment of different ailments such as stomach and ear pain.

In addition, the rosehips are also used for making tea, soups, marmalade, yoghurt and other beverages. They are known for high content of vitamins B, C and E, polyphenols, and carotenoids (Lockwood, 2012). The oil extracted from the seeds of rosehips contains essential oils, linoleic acid (omega 3) and linolenic acid (omega 6) and is used globally for treating eczema, as anti-anxiety remedy and as cosmetic oil (Lockwood, 2012).

Though various propagation methods such as budding, grafting and cuttings (Oliver, 2009) for *Rosa* species exist, seed propagation techniques which require less labour with no special expertise are lacking. This is because of the presence of deep seed dormancy of *R. rubiginosa* which make it difficult to establish commercial plantations from seeds. Seed dormancy plays a critical role in ensuring successful development of seeds in readiness for germination since it postpones the seed germination until all factors are appropriate (Graeber *et al.,* 2012).

Despite this survival advantage, excessive dormancy can be problematic (Hofmann, 2014). During maturation stage, it is common that most seeds of different plants enter dormancy (Wang *et al.,* 2013). The seeds of *R. rubiginosa* enter a deep dormancy at

maturity (Meyer, 2008). The effect of deep dormancy is the prolonged delay in germination as well as excessively low germination percentages (Kazaz *et al.,* 2010). Meyer, (2008) observed that rose seeds have a thick, hard pericarp and they do not swell when placed in water, and the presence of this hard pericarp makes the seeds to have minimal permeability to water. In addition, deep dormancy in *R. rubiginosa* is also associated with the presence of higher concentrations of an inhibiting hormone called abscisic acid (ABA) in the pericarp and testa (Hartmann *et al.,* 2002).

Different pre-treatment methods have been tested for their potential in breaking the dormancy of *R. canina* seeds and other *Rosa* species and some of them include macerating enzymes (Yambe *et al.,* 1992), seed scarification with sulphuric acid (Bhanuprakash *et al.,* 2004), priming with gibberellic acid (Hoşafçi *et al.,* 2005), stratification (Zlesak, 2007) and hot water pre-treatment (Younis *et al.,* 2007). However, these methods gave unsatisfactory germination percentages (Haouala *et al.,* 2013). Thus the main aim of this study was to evaluate scarification and stratification techniques in breaking seed dormancy and develop regeneration and transformation protocols of *R. rubiginosa*.

1.1   Statement of the Problem

Although seed propagation could be the best way of establishing large plantations of *R. rubiginosa*, seed dormancy presents a major bottleneck. The seed dormancy in *R. rubiginosa* is due to both mechanical and physiological factors. Mechanical dormancy is attributed to the hardness of the thick pericarp that prevents the expansion of the embryo, gas exchange and imbibition of water by the seeds (Bradbeer, 1988). On the other hand, physiological dormancy is mainly attributed to high concentration of ABA in the pericarp and testa of the seeds (Hartmann *et al.,* 2002). Moreover, there is no protocol for *in vitro*

regeneration of *R. rubiginosa* which limits its large scale propagation and improvement of its traits through biotechnological tools.

1.2 Justification

The seeds of *R. rubiginosa* have deep seed dormancy resulting in low or no germination when the seeds are planted (Meyer, 2008). Therefore there is a need to develop a protocol that can break seed dormancy of this plant. In the present study, hot water treatment, acid scarification, warm and cold stratification techniques were used because their simplicity and cheapness with no requirements for complex equipment. There is a need for development of efficient and reproducible regeneration protocol for future application of biotechnological tools to *R. rubiginosa*. Therefore successful development of an efficient regeneration protocol for *R. rubiginosa* would result in large scale production of its seedlings to help establish its plantations in order to help alleviate poverty in rural communities.

Regeneration requirements for roses are species specific because of high heterozygosity and polyploidy (Ambros *et al.,* 2016) as such there was a need to develop a regeneration protocol specifically for *R. rubiginosa*. Therefore this study was carried out to evaluate different scarification and stratification techniques in breaking seed dormancy of *R. rubiginosa*, also to develop regeneration protocol for *R. rubiginosa* using leaf and nodal explants. The results from this thesis work will be useful to researchers and farmers.

1.3 Objectives

1.3.1 General Objective

To evaluate scarification and stratification techniques in breaking seed dormancy and to develop a regeneration protocol for *R. rubiginosa*.

1.3.2 Specific Objectives

1. To determine efficacy of hot water treatment (stratification) alone or in combination with sulphuric acid scarification in breaking seed dormancy of *R. rubiginosa*.

2. To develop a regeneration protocol for *R. rubiginosa* using leaf and nodal explants

1.4 Significance of the Study

Rosa species is known for its recalcitrant nature during regeneration and this presents a major problem for application of commercial tissue culture programs and genetic engineering to improve its traits. *R. rubiginosa* is mainly found distributed in rural areas of Lesotho and the communities around such places earn their livelihoods from it by harvesting and selling it to the local companies which process it into various finished products such as rosehip powder, rosehip shells, joint assist, rosehip tea, rosehip tea blend and rosehip oil extracted from the seeds.

However, the potential benefits from *R. rubiginosa* as mentioned by Werlemark (2009), are beyond the forth mentioned since other parts of the plant are yet to be exploited. Thus the breakthrough in developing both regeneration protocol of *R. rubiginosa* or breaking its seed dormancy will help these rural communities to establish their own plantations and generate more income hence alleviate poverty. Thus success of this project will improve the living standards of people in Lesotho in particular those in rural areas.

Lesotho is suffering from climate change effects characterized by prolonged droughts and short growing seasons (FAO, 2012) thus limiting the good yields of staple crops like maize and sorghum from which most families earn their livelihoods. This is in addition, to the fact that Lesotho is a mountainous country with only 12% arable land (CIA, 2013) and the advantage of *R. rubiginosa* is that it grows optimally on the mountainous parts of Lesotho and it is also drought tolerant. Thus this crop can provide an alternative source of income to the most families of Lesotho.

1.5   Scope of the Study

The scope of this study was to investigate the effectiveness of hot water treatment, sulphuric acid scarification, warm- and cold stratification techniques in breaking seed dormancy of *R. rubiginosa*. The effectiveness of the tests used to break seed dormancy was tested by performing germination tests on petri dishes containing double layer filter papers under controlled environment in the growth room at $25\pm1\,^{\circ}\mathrm{C}$ in complete darkness. Moreover, the scope of this study was to develop a protocol for micropropagation of *R. rubiginosa* through both direct and indirect regeneration using nodal and leaf explants.

CHAPTER TWO: LITERATURE REVIEW

2.0   Botany, Classification and Ecological Requirements

R. rubiginosa is a deciduous plant, characterized by prickly and bristled shoots at the end of spring and it belongs to *Rosaceae* family (Horn, 1992). Its flowers which last only for few days are pollinated by flies and bees and each flower consists of 50-200 stamens and 5-150 pistils contained in receptacle (Rogers, 1992). It is this receptacle that develops into a fleshy, berrylike rosehip. During maturation process, the rosehip changes color from green to red.

The prickles on the stems can be up to 20 mm long and are usually hooked and some species have smaller prickles interspersed with the large ones (Werlemark, 2009). It grows well in moist soils and preferably on rich, slightly acidic or neutral soils. It also grows optimally on heavy clay soils that result in production of more flowers. It prefers full sun exposure and when grown in deep shade areas it fails to bear flowers and fruits (Rogers, 1992).

Figure 1: *Rosa rubiginosa* plant bearing flowers (left) and bearing the rosehips (right) (Werlmark, 2009).

2.1   Economic Importance *R. rubiginosa*

Various components of the *R. rubiginosa* plant have various commercial applications. For instance, to start with, rosehips (fleshy red part) are used in medicine for treating different ailments including stomach pains and ear problems (Werlemark, 2009). Rosehips are rich in polyphenols, vitamins B, C and E, carotenoids and the minerals like potassium and phosphorus (Lockwood, 2012). Due to the above mentioned properties in addition to their aroma, rosehips are used for making tea, marmalade, yoghurt and other beverages including soup for use as a snack and dessert soup (Werlemark, 2009).

Figure 2: Products from rosehips and seeds. Rosehip powder used in healing joint pains (A), rosehip tea (B), rosehip tea blend(C) and rosehip oil extracted from the seeds (D). Picture provided by "The Rosehip Company, Lesotho" (www.therosehipcompany.co.ls).

On the other hand, the oil extracted from the seeds which is used to cure different skin problems is characterized with high content of essential oils, omega-3 (linoleic acid) and

omega-6 (linolenic acid) (Kole, 2011). Furthermore, the petals from the flowers are used in medicine, food as coloring agent and in perfumery industry as fragrant additives (Williams, 2013).

2.2   Seed Dormancy

The stages of development for most plants follow the sequence: seed, germination, vegetative and reproductive phase as final stage and these stages are tightly controlled in part by seed dormancy to ascertain that each stage occur at most optimal conditions (Graeber *et al.,* 2012). Seed dormancy is therefore an adaptation mechanism for seeds and it is classified as physiological, morphological, morpho-physiological, physical and combinational dormancy (Baskin and Baskin, 2004).

Seeds that acquire dormancy while still on the parental plants are known to have primary dormancy while seeds acquiring dormancy at maturity due to unfavorable conditions are known to bear secondary dormancy (Bewley and Black, 1994). Thus, seed dormancy is a vital component of every plant's fitness and so it is directly influenced both by the genetic factors and the environment that a plant is exposed to (Huang *et al*. 2010).

Therefore the major types of dormancy are primary and secondary dormancy (Geneve, 2005). Primary dormancy could be as a result of exogenous, endogenous and combinational dormancy whereas secondary dormancy could be as a result of thermo- and conditional dormancy (Hartmann *et al.,* 2010). The major difference between the two is primarily the time at which the seeds acquire the dormancy. For instance, for primary seed dormancy, the seeds acquire the dormancy before they are shed from their parent plant (Chahtane *et al*., 2017). In contrast, non-dormant seeds which get exposed to unfavorable

environmental conditions acquire seed dormancy to prevent risking germination under unfavorable environment and this is referred to as secondary dormancy (Hartmann *et al.,* 2010).

2.3   Hormonal Regulation of Seed Dormancy

It has been proved experimentally that hormones that control both growth inhibiting and germination are involved directly in the regulation of seed development, dormancy and germination (Hartmann *et al.*, 2010). Different studies have been conducted to give the evidence of the role hormones play in seed dormancy and several experiments on correlations between hormone concentrations with specific developmental stages, effects of applied hormones, mutants for hormone production or perception, and genome-wide microarray analysis have been established (Finkelstein *et al.,* 2008).

The major hormones that regulate seed dormancy are mainly abscisic acid (ABA) and gibberellins (GA). The main role of ABA is to initiate and maintain seed dormancy whereas in contrast, the function of GA is to activate seed germination and so the hormonal ratio between ABA and GA determines the fate of the seeds (Finch-Savage and Leubner-Metzger, 2006).

The principal role of ABA is inhibiting precocious germination of the seeds and it is mainly responsible for primary seed dormancy since its concentration increases as the seed matures (Kermode, 2005). The research shows that seeds characterized with lower concentrations of ABA display minimal primary seed dormancy as compared to those with higher amounts of ABA that show high primary seed dormancy (Groot and Karssen, 1992). The amount of accumulated ABA in a seed vary at different parts of the seed as it

has been shown that in a peach seed, ABA was highly concentrated in the seed coat with lower amounts in the cotyledons (Hartmann *et al.*, 2010).

ABA acts by inhibiting GA biosynthesis enzymes thereby maintaining seed dormancy (Seo *et al.,* 2009). On the other hand, GA control and promote seed germination (Peng and Harberd, 2002) by activating enzymes responsible for weakening the seed coat or endosperm, stimulating mobilization of seed storage reserves and activating the expansion of the embryo cells (Finch-Savage and Leubner-Metzger, 2006).

In contrast to ABA, GA is found in higher quantities during the early stages of the seed development but decreases as the seed matures especially in dicotyledonous seeds (Kucera *et al.,* 2005). According to Yamauchi *et al.*, (2004) during cold stratification, GA is either biosynthesized or converted to an active form. Finch- Savage *et al.,* (2006), reported that GA is deactivated in dormant seeds of *Arabidopsis* whereas in non-dormant seeds, there is a high expression of genes responsible for the biosynthesis of endogenous GA.

Ethylene (Et) does not limit germination of the seeds but it determines the rate of seed germination and the seed vigor (Matilla and Matilla, 2008). In addition, significant increase of Et during radicle protrusion was reported and so it is suggested that Et is involved in breaking seed dormancy in some seed species (Kepczynski and Kepczynska, 1997).

It has been shown that there is a significant antagonism between ethylene and seed sensitivity to ABA and it is assumed that at least one mechanism for ethylene-induced dormancy release is reducing the embryos' sensitivity to ABA (Cheng *et al.*, 2009). Ethylene production and application has been implicated as a mechanism to alleviate

thermo-dormancy for chickpea (Gallardo *et al.,* 1996) and lettuce (Huang and Khan, 1992).

Cytokinins are known to be involved in seed germination by permitting GA to function (Hartmann *et al.*, 2010). Moreover, cytokinins are suggested to have an antagonistic interaction with ABA which leads to increased production of ethylene subsequently reducing sensitivity of seeds to ABA (Matilla, 2000). Another hormones regulating seed dormancy are brassinosteriods which are capable of releasing seed dormancy of mutant seeds with impaired synthesis of GA (Leubner- Metzger, 2001).

Butenolide is an active compound derived from smoke concentrate (Daws *et al.,* 2008). Its discovery has resulted in the synthesis of a new plant growth regulating hormone known as karrikins (Nelson *et al.,* 2009), Karrikins act by interacting with ABA and GA (Daws *et al.,* 2007) and have proofed effective in releasing dormancy of seeds in approximately 1,200 species (Dixon *et al.,* 2009).

2.4 Mechanism of Seed Dormancy in *Rosa* Species

The seeds of *Rosa* species bear both physical and physiological dormancy mechanisms (Werlemark, 2009). Physical dormancy is due to thick, hard pericarp contributing to limited imbibition of water by the seeds, limited gaseous exchange, inaccessibility of embryo to the food reserves and reduced expansion of the embryo (Meyer, 2008). Zlesak (2007) reported that the variation in germination of rose seeds depends on genotype, prevailing temperatures during seed development, maturity level of the seeds during harvest, and pre-treatment techniques used before sowing.

For instance, it was shown that the seeds of hybrid tea rose harvested after exposure to

high temperatures and more light gave higher germination in comparison to those harvested during low temperature and less light (Meyer, 2008). Moreover, it has been shown that the extent of embryo development and thickness of the pericarp are relatively affected by temperature (Gudin *et al.,* 1990)

Gudin *et al.,* (1990) investigated the effects of temperature and genetic origin on the development of ovule and thickness of the pericarp of rose seeds. They observed that the overall effect of endocarp barrier is defined during early stages of seed development and they concluded that relationship between the speed of seed development and the thickness of the endocarp is inversely proportional.

Consequently, their results revealed that the slower the rate of seed development, the higher the thickness of the endocarp barrier and so the higher the effect of mechanical seed dormancy. Despite the important role of genetic origin with regard to embryo development, during seed development, warmer temperature is known to promote well developed embryo whereas cold temperature may results to under developed embryos or withered embryos (Gudin *et al.*, 1990).

In contrary, physiological seed dormancy in *Rosa* species is due to presence of high ABA concentration in the seed testa and pericarp (Werlemark, 2009). Hartmann *et al.,* (2002) estimated ABA to be 10 to 1000 fold higher than in seeds of other plants. The presence of high ABA in the seed coat inhibits the activity of GA which is important for activating germination of the seed (Gómez-Cadenas *et al.,* 2001).

2.5   Techniques used to Break Seed Dormancy of Roses

Breaking seed dormancy is a complex process resulting in alterations in the pericarp, testa and embryo of the seed (Nadeem *et al.,* 2013). As indicated earlier seed dormancy in *Rosa* species is of two-fold: mechanical and physiological and many researchers have tried different techniques to break seed dormancy in *Rosa* species.

These techniques include scarification of seeds with sulphuric acid and cold stratification (Nadeem *et al.,* 2013); microbial inoculation of the seeds (Kazaz *et al.,* 2010); seeds treatment with macerating enzymes (Yambe *et al.,* 1992); hot water treatment (Younis *et al.,* 2007); seeds priming with GA (Hoşafçi *et al.,* 2005); vacuum-infiltration of the seeds with GA or benzyladenine; warm plus cold stratification (Zlesak, 2007). However, lower success rates have been reported. During cold stratification, the concentration of ABA decreases while that of GA increases (Lee and Looney, 1978).

It is also assumed that cold stratification activates embryo to initiate germination process by increase the production of some growth-promoting substances including GA which promotes germination (Chebouti-Meziou *et al.,* 2014). The overall effect of seed scarification is weakening seed tegument which allows water imbibition (Younis *et al.,* 2007). This results in activation of germination of the seed due to activation of metabolic reactions of the embryo and the cotyledons (Ahoton *et al.,* 2009).

2.6   *In vitro* Regeneration

Efficient *in vitro* plant propagation protocol is the key for application of biotechnological tools including clonal propagation, mutation breeding, and genetic transformation techniques (Debener and Hibrand-Saint Oyant, 2009). The fact that tissue culture is done

in vitro it allows easy manipulation of the environment and growth medium leading to production of new plants. The composition of the medium, particularly the plant hormones and the nitrogen source has profound effect on the morphology of the tissues that grow from the initial explant (Desai *et al*., 2015).

For example, high concentrations of auxins stimulate cell expansion leading to proliferation of the roots, while high concentrations of cytokinins initiate shoots by stimulating deoxyribonucleic acid (DNA) synthesis and increased cell division of undifferentiated cells (Jacqmard *et al*., 1995). Thus, a balance between auxins and cytokinins results in production of undifferentiated mass of cells known as callus, because both cell division and cell expansion occur in the actively dividing tissue (Perianez-Rodriguez *et al*., 2014).

However, the morphology of the outgrowth is determined by the plant species and the composition of the medium. As cultures grow, pieces are cut off and sub-cultured to a new medium to allow for growth of the culture. After emergence of shoots, the plantlets are transferred to root inducing medium that has auxins leading to production of full plants which can be eventually transferred to potting soil for further growth in the greenhouse as normal plants.

Any part of the plant which is excised for the purpose of initiating tissue culture is known as explant (Purohit, 2012). The most suitable part to use for initiating tissue culture is depended on the plant species and the objective of the study. The response of the explant in culture medium will depend on the genotype of the donor plant, plant species, age of the plant, developmental stage of the explant and physiological stage of the explant and

donor plant (Bajaj, 2012).

Generally, most plants are regenerated from actively growing plant tissue (Tian *et al.*, 2008). Regeneration of plants *in vitro* is a preliminary phase for plant reproduction and transformation (Pati *et al.,* 2006). However, various factors affect success of regeneration of roses including presence or absence of petioles, temperature, genotype and exudation of phenolic compounds (Skirvin *et al.* 1990). Phenolic compounds cause medium browning thereby making it autotoxic to the plant (Thomas, 2008).

Media browning develops when enzymes and other compounds exuded from the cut end of the plant react with compounds in the medium (Ahmad *et al.*, 2013). Addition of oxidative inhibitors such as polyvinylpyrrolidone (PVP), ascorbic acid (AA) or activated charcoal in the medium prevents browning formation (Rani and Dantu, 2016). Thus, media composition is a very important factor for successful regeneration of the rose plant.

Shoot proliferation *in vitro* is largely the result of cytokinin in the medium (Skirvin *et al.,* 1990). According to Bressan *et al.* (1982), benzyladenine (BA) at low concentrations resulted in good proliferation rate for "Golden Glow", but not for "Improved Blaze". For rooting, most rose species are known to root easily *in vitro* even spontaneously on proliferation medium (Canli and Skirvin, 2003). Rooting medium commonly consists of MS mineral salts with or without growth regulators (Skirvin *et al.,* 1990).

The most commonly used auxins for rooting of roses are naphthaleneacetic acid (NAA), Indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) (Pati *et al.*, 2006). Many roses root well in half or quarter strength MS salt concentrations and it was reported by Arnold *et al.* (1995) that as the concentration of salt increased, the amount of IBA and NAA

required for optimum root growth also increased in *R. kordesii* cv. *Champlain*.

2.7   Genetic Transformation of Roses

Genetic engineering techniques can be used to improve the traits of roses such as biotic and abiotic stress resistance, scent improvement, high yield of rosehips, less thorns of the rose plant, high flowering, plant architecture and flower color (Folta and Gardiner, 2009). Genetic transformation requires availability of reliable protocols for *in vitro* regeneration, transfer of genes and selection and regeneration of transformed plants (Li *et al*., 2002).

The techniques used for the transformation of roses are based on biological vectors such as *Agrobacterium*-mediated transformation and non-biological vectors such as particle bombardment or biolistic transformation (Marchant *et al*., 1997; Li *et al*., 2002). However, rose is considered to be recalcitrant for genetic manipulation due to low rates of transformation and regeneration (Folta and Gardiner, 2009).

Plant transformation process involves transfer of target gene into the plant's genome, *in vitro* regeneration of the explant into a full plant, selection of transgenic plants and confirmation of their transgenic nature (Kim *et al*., 2004). As a pre-requisite for the development of an effective transformation protocol the choice of an explant is the key. The selection of an appropriate explant is a critical factor when conducting transformation and regeneration experiments since different explants have different capabilities for transformation and regeneration (Folta and Gardiner, 2009).

Majority of published protocols for transformation of *Rosa* plants are based on *Agrobacterium*-mediated technique except for Marchant *et al.,* (1997) who used biolistic transformation technique. For the previous *Agrobacterium*-mediated transformation

protocols various *Agrobacterium* strains C58C1, AGL0, GV3101, EHA105, GV2260, and LBA4404 have been used with success. Kim *et al.,* (2004) showed that in order to increase efficient transformation of *Rosa* plants more *vir*E/*vir*G genes are required. In addition, Firoozabady and Moy, (2004) reported that the presence of acetosyringone in the transformation medium may increase the transformation efficiency.

Previous reports used binary transformation vectors containing neomycin phosphorus transferase gene (*nptII*) for selection based on kanamycin resistance (Debener and Hibrand-Saint Oyant, 2009). Different researchers used different concentrations kanamycin for selection of successful transformants: for selection of transgenic embryogenic callus, Li *et al.,* (2002) used 50 $mg^.L^{-1}$ while for selection of secondary somatic embryos 100 $mg^.L^{-1}$ was used. In contrast, Derks *et al.,* (1995) used 300 $mg^.L^{-1}$ selecting for putative transformed embryogenic callus while van der Salm *et al.,* (1998) utilized 5 $mg^.L^{-1}$ for selection of root formation.

Improved transformation protocol for different cultivars of roses was developed by Condiffe *et al.,* (2003). In their study, *Agrobacterium tumefaciens*-mediated transformation of embryogenic callus from a number of rose cultivars was optimized using beta-glocuronidase (gus; uidA) reporter gene. An optimized protocol was used to deliver transgenes into cultivars for improving flower production, disease resistance or re-introducing scent into cultivars that did not have it (Condiliffe *et al.,* 2003).

Zakizadeh *et al.,* (2013) developed a transformation protocol for miniature potted rose (*R. hybrida cv*. Linda) using P$_{SAG12}$-*ipt* gene. Transgenic *R. hybrida cv*. Linda plants were obtained through transformation with *A. tumefaciens* harboring the binary vector pSG529(+) containing P$_{SAG12}$-ipt construct. Other strains of *Agrobacterium* namely

AGL1, GV3850 and LBA4404 containing P$_{35S}$-INT β-glucuronidase (GUS) gene have been used for transformation of embryogenic callus (Zakizadeh *et al.,* 2013).

Vergne *et al.,* (2010), reported a successful protocol for somatic embryogenesis and transformation of the diploid *R. chinensis* cv Old Blush. Somatic embryogenesis was induced from *in vitro*-derived leaf explants of *R. chinensis* cultivar (cv) Old Blush and transformation was achieved through *A. tumefaciens*-mediated transformation consisting of GUS reporter gene (Vergne *et al.,* 2010). Marchant *et al.,* (1997) reported Biolistic Transformation of Rose (*R. hybrida* L.). They developed a reproducible method for the biolistic transformation and regeneration of transgenic plants from embryogenic callus of rose (*R. hybrida* L.) cv. Glad Tidings. DNA delivery was optimized using the GUS gene.

Katsumoto *et al.,* (2007) successfully engineered rose flavonoid biosynthetic pathway resulting in blue-hued flowers due to accumulating delphinidin in the petals of the flowers. Dohm *et al.,* (2002), successfully transformed roses with genes for antifungal proteins to reduce their susceptibility to fungal diseases. This was achieved through overexpression of genes that regulate antifungal proteins using *Agrobacterium* mediated gene transfer of somatic embryos of garden rose cultivars Heckenzauber and Pariser Charme which showed 60% reduced infection susceptibility against black spot.

Pourhosseini *et al.,* (2012), reported an *Agrobacterium*-mediated transformation of *chitinase* gene in *R. damascene* cv. Ghamsar. Axillary buds of *R. damascene* cv. Ghamsar were inoculated with *Agrobacterium* under vacuum condition after treatment with carborandum solution. *A. tumefaciens* strain LBA4404 carrying pBI121 plasmid containing *chitinase* gene under control of CaMV35S promoter and nptII selectable

marker gene was used.

CHAPTER THREE: MATERIALS AND METHODS

3.1   Sample Collection

The cuttings of *R. rubiginosa* L. were collected in October from the wild in Maseru, Lesotho and shipped to Kenya enclosed in polythene paper bag. The sample collection was done using a random technique. The seeds of recent harvest of a total quantity of 2 kg were obtained from The Rosehip Company, Mohale's Hoek, Lesotho.

3.2   Pretreatment of the Seeds

The seeds of treatments T0 (control) and T1 were first treated for 1 min with concentrated sulphuric acid. Then the seeds were transferred to hot were treatment for 0 (T0, control) and 5 min (T1). Then the seeds for treatments T2 - T5 were treated with hot water at 97℃. The seeds from all the treatments (T0 − T5) were transferred to cold stratification at 5℃. The portion of T0 and T1 seeds was also kept at warm conditions at 25℃. The seeds were kept in moist vermiculite medium.

Table 1: Summary of hot water treatment in combination with acid scarification, warm and cold stratification techniques

Label	Acid Scarification (Conc. H2SO4)	Hot Water Treatment (97℃)	Cold Stratification (5℃)	Warm Stratification (25℃)
T0	1 min	0 (control)	22 weeks	22 weeks
T1	1 min	5 min	22 weeks	23 weeks
T2	0	15 min	23 weeks	0
T3	0	30 min	24 weeks	0
T4	0	45 min	25 weeks	0
T5	0	50 min	26 weeks	0

In another treatment, the seeds were treated with hot water (97℃) for 5 min followed by soaking at various concentrations of GA₃ for 1 and 10 days in a shaker incubator set at 100 rotations per minute (rpm). Then after each treatment, the treated seeds were transferred to 7 weeks cold stratification at 5℃ in sterilized river sand. The summary is shown in table 2.

Table 2: Summary of hot water treatment plus GA3 priming and cold stratification

Hot Water Treatment (97℃)	Concentration of GA3 (mg·L⁻¹)	Duration (Days)
5 min	0	1
		10
	50	1
		10
	250	1
		10
	500	1
		10
	1000	1
		10
	2000	1
		10

3.3 Seeds Sterilization and Germination Test

The seeds treated as mentioned in tables 1 and 2, were sterilized as follows: immersed in 20% jik containing 3.85% NaClO for 10 minutes. The seeds were then rinsed three times with sterile distilled water. Finally the seeds were placed for 10 min in 70% ethanol followed by thorough rinsing with sterile distilled water. Then the germination test was performed as follows:

A total of 100 seeds were placed in petri dishes with a double layer filter papers moistened with distilled water. The petri dishes were sealed with Parafilm and placed in the growth room for germination test at 25°C in darkness. Each treatment had a total of 300 seeds divided into three

replicates of 100 seeds per plate. The control group consisted of seeds that were not treated with any of the above mentioned treatments.
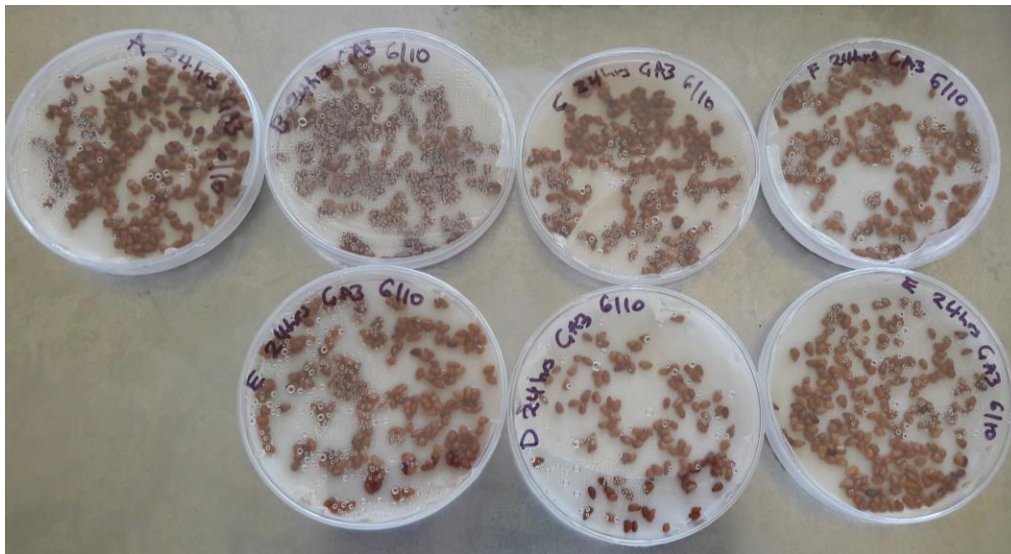
Figure 3: Germination Test. Germination test of seeds treated with hot water and primed with GA3 for 24 hours: untreated seeds (A), hot water treated seeds for 5 min (B), hot water treated seeds for 5 min and primed with 0 mgL^{-1} GA3 (C), hot water treated seeds for 5 min and primed with 50 mgL^{-1} GA3 (D), hot water treated seeds for 5 min and primed with 250 mgL^{-1} GA3 (E) and hot water treated seeds for 5 min and primed with 500 mgL^{-1} GA3 (F).

3.4   Dye Penetration Test

Dye penetration test was done to test the effectiveness of hot water treatment in promoting seed imbibition. The test was done according to Orozco-Segovia *et al.*, (2007). Briefly, the seeds treated with hot water for 5 minutes were soaked in 1% red stain dye in darkness at room temperature. After 24 hours of staining, the seeds were removed to determine dye penetration. The control consisted of seeds not pretreated with hot water.

3.5 Water Imbibition Test

The initial mass of forty seeds was measured in triplicates. The weighted seeds were subsequently treated with hot water at 97℃ for 0 (control) or 5 minutes. Following the treatment, the seeds were dried on a blotting paper towel and weighed again. Then the seeds were submerged on agar medium (3 g agar/L of water) with forty seeds per bottle and placed in complete darkness. The change in weight was recorded by measuring the mass of the seeds from each bottle at intervals of 3 days till the 9th day. Each time the seeds were taken from the agar, they were blotted with a paper towel, immediately weighed and returned to the agar medium. In order to determine the percentage of imbibed water, the following equation was used:

$$Water\ imbibition\ (\%) = \frac{(Final\ mass - Initial\ mass)}{Initial\ mass} \times 100\%$$

Both final and initial mass represents an average of 40 seeds in replicates of three measured at day 0, day 3, day 6 and day 9 . Day 0 is the first day.

3.6 *In vitro* regeneration for Rosa rubiginosa

3.6.1 Sample Collection

For direct regeneration using nodal explants, the cuttings of *R. rubiginosa* were collected in October from the wild in Maseru, Lesotho and shipped to Kenya with DHL cargo enclosed in polythene paper bag and covered with a DHL plastic bag. The cuttings were received on the third day and used for tissue culture on the fourth day. For indirect regeneration, leaf explants were collected from 4-weeks old *in vitro* plants from the growth room.

3.6.2   Explant preparation and sterilization

The nodal explants were prepared by first removing thorns from the stems. Then about 2 cm long cuttings with a single node were cut from stems. The leaf explants were prepared by removing the three top leaves on the branches of *in vitro* grown plants using a scalpel blade. The leaf explants were cut once near the point of petiole attachment to the leaf. For sterilization, three different fungicides were used namely Ridomil® Gold™ MZ (RG-MZ) and Antracol® WP 70 (AWP-70), at a concentration of 20 mg·ml^{-1} and Azoxy Top 325SC (AT-325SC) at 25% concentration. In all fungicide solutions 3 drops of tween 20 were added.

The nodal explants were sterilized by first washing for 30 minutes in running tap water followed with soaking for 30 minutes in 10% savlon antiseptic. Then explants were rinsed 3 times with sterile distilled water (SDW) before transferring to 2% NaClO solution for 20 minutes. After rinsing four times with SDW explants were soaked in 70% ethanol for 10 minutes followed with four times rinsing with SDW before use in the experiments. The leaf explants from *in vitro* grown cultures were already sterile therefore were used directly without sterilization.

3.6.3   Media Preparation and Culture Conditions

In all the treatments, the pH of Murashige and Skoog (MS) medium was adjusted to a range of 5.7-5.8 and the medium contained 3 g/l gelrite. The media was autoclaved at 121°C with pressure of 15 lbs for 20 minutes. The cut end of the nodal explants were placed in contact with the medium in up right orientation for all treatments except in a treatment for determination of effect of explant orientation where some explants were cultured in a horizontal orientation. Each treatment was done in triplicate. For all the

treatments the cultures were placed in the presence of light provided by fluorescent bulbs with 50 mEm^{-2}/s intensity in the tissue culture room at $25\pm1^{o}C$ except for callus and rhizoid induction where the incubation was performed in complete darkness.

Table 3: Summary of media treatments for sterilization, shooting, callus and rhizoid induction

Media	Function of Media	Treatments (mg·L^{-1})	Media Properties
T1	Developing sterilization protocol	-	Full strength MS medium devoid of hormones
T2	Shoot induction from nodal explants	0, 0.5, 2.5, 5.0, 10	Full strength MS medium with various treatments of ascorbic acid
T3	Shoot induction from nodal explants	0, 0.5, 2.5, 5.0, 10	Full strength MS medium with various treatments of activated charcoal
T4	Shoot induction from nodal explants	0, 0.5, 2.5, 5.0, 10	Full strength MS medium with various treatments of BAP
T5	Shoot induction from callus	0.0, 0.4, 0.5, 1.0, 2.0 3.0 and 0.1 + 0.5, 0.5 + 0.5, 1 + 0.5, 1.5 + 0.5, 2 + 0.5	Full strength MS medium with various treatments of BAP and full strength MS medium with various treatments of BAP in combination with 0.5 mg·L^{-1} NAA and vice versa.
T6	Determining effect of explant orientation on shoot induction	0, 1.0, 2.0, 3.0, 4.0	Full strength MS medium various treatments of BAP
T7	Callus induction from leaf explants	0, 1.0, 1.5, 2.0, 2.5	Full strength MS medium with various treatments of 2,4-D
T8	Rhizoid induction from callus	0, 1.5, 3.0, 6.0	Full strength MS medium with various treatments of 2,4-D

Table 4: Media treatments for root induction

Trial	Treatments (mg/L)	Media Properties
1	0, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35	Full strength MS medium with 3% sucrose, 0.3% gelrite and various treatments of NAA
2	0, 1, 2, 3	Full strength MS medium with 3% sucrose, 0.3% gelrite and 2.0 mgL^{-1} BAP plus various treatments of IBA
3	0, 1, 2, 3	Full strength MS medium with 3% sucrose, 0.3% gelrite and 2.0 mgL^{-1} BAP plus various treatments of IAA
4	50, 250, 500	Full strength liquid MS with various treatments of NAA. Shoots kept for 24hrs before cultured to full strength MS with 3% sucrose and 0.3% gelrite
5	50, 250, 500	Full strength liquid MS with various treatments of IBA. Shoots kept for 24hrs before cultured to full strength MS with 3% sucrose and 0.3% gelrite
6	50, 250, 500	Full strength liquid MS with various treatments of IAA. Shoots kept for 24hrs before cultured to full strength MS with 3% sucrose and 0.3% gelrite
7	40 000	Full strength MS medium with 40 gL^{-1} sucrose and 0.3% gelrite
8	20 000	Full strength MS medium with 20 gL^{-1} sucrose and 0.3% gelrite
9	15 000	Half strength MS medium with 15 gL^{-1} sucrose and 0.15% gelrite
10	7 500	Half strength MS medium with 7.5 gL^{-1} sucrose and 0.15% gelrite
11	0, 0.1, 0.2, 0.3, 0.4, 0.5	Full-strength MS medium with 6.25 mgL^{-1} ascorbic acid, 2% sucrose, 0.15% gelrite and various treatments of NAA
12	0, 0.1, 0.2, 0.3, 0.4, 0.5	Full-strength MS medium with 6.25 mgL^{-1} ascorbic acid, 2% sucrose, 0.15% gelrite and various treatments of IBA
14	0, 0.1, 0.2, 0.3, 0.4, 0.5	Full-strength MS medium with 6.25 mgL^{-1} ascorbic acid, 2% sucrose, 0.15% gelrite and various treatments of IAA
15	0.5, 1.0, 1.5	Full-strength MS medium with 3% sucrose, 0.3% gelrite, 0.5 mgL^{-1} NAA and IBA and various treatments of IAA
16	0.1, 0.25, 0.5	Full-strength MS medium with 3% sucrose, 0.3% gelrite, 3 mgL^{-1} BAP and various treatments of IAA
17	0, 0.25, 0.5	Full-strength MS medium with 3% sucrose, 0.3% gelrite, 3 mgL^{-1} IAA and various treatments of NAA
18	0, 0.25, 0.5	Full-strength MS medium with 3% sucrose, 0.3% gelrite, 6 mgL^{-1} IAA and various treatments of NAA

CHAPTER FOUR: RESULTS

4.1   Breaking Seed Dormancy

All the treatments used for breaking seed dormancy were not effective. In this study, the seeds treated with hot water for 5 minutes were also treated with concentrated sulphuric acid for 60 seconds however even the use sulphuric acid did not yield any positive results.

4.2   Dye Penetration Test

The two ends of the seeds of *R. rubiginosa* are different in terms of size; one end is sharp and small and while the other is bigger and blunt. After soaking the seeds for 24 hours in 1% solution of congo red, the seeds showed the red stain sparingly distributed on the surface of the seeds (Figure 4).
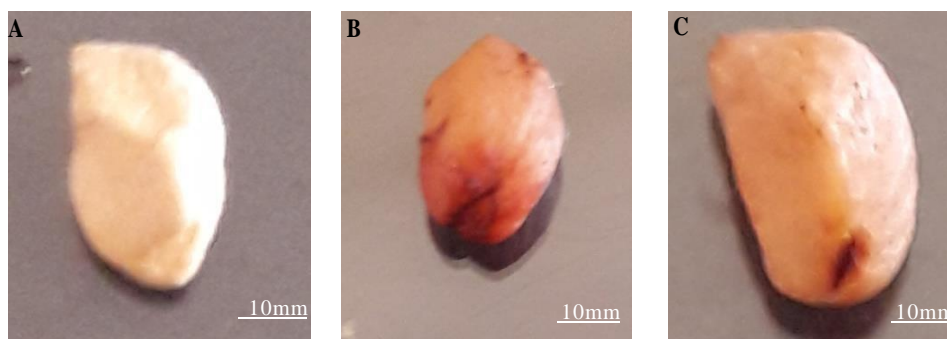
Figure 4: Dye penetration test results on *R. rubiginosa* seeds stained with 1% congo red showing the seed before staining (A), boiled and stained (B) and un-boiled stained seed (C).

The principle of both treatments (hot water and sulphuric acid) is that they promote germination by weakening the hard seed coat of the seed thereby allowing imbibition of the water into the seed. The seeds treated for 5 minutes with hot water treatment were used to study the change in their imbibition rate. Based on the differences on weight of the seeds before and after boiling, the highest amount of imbibed water by the seeds was

calculated as 28%. However, when performing germination test the seeds sown on petri dishes containing moistened filter papers did not germinate.

Interestingly, all the stained seeds showed one similar staining pattern, the dye was more concentrated on the bigger, blunt end of the seeds (Figure 5). However, when these seeds were cut longitudinally to observe how far the dye had penetrated, all the seeds did not show any red color and no differences were observed from the control (unstained seeds) (Figure 5). This implied that the dye did not penetrate the seeds but was just on the seed coat.
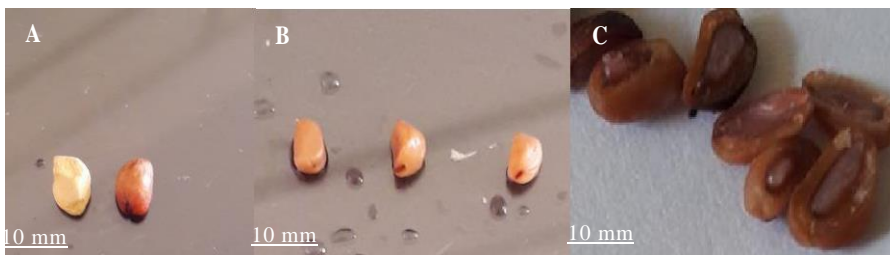
Figure 5: Staining pattern of *R. rubiginosa* seeds after soaking in 1% congo red for 24 hours. Unstained- and stained-seed of *R. rubiginosa* blunt end (A), staining pattern of *R. rubiginosa* seeds after 24 hours pointed end (B) and longitudinal section of *R. rubiginosa* seeds showing no dye penetration into seeds cotyledons (C).

4.3   Water Uptake by the Seeds

The treatment seeds showed a rapid increase in weight following hot water treatment. However, after 3 days of imbibition studies in agar medium, the moisture content of the seeds remained unchanged. Control seeds showed increased water content on the first 3 days of imbibition studies after which water content remained unchanged (Figure 6).
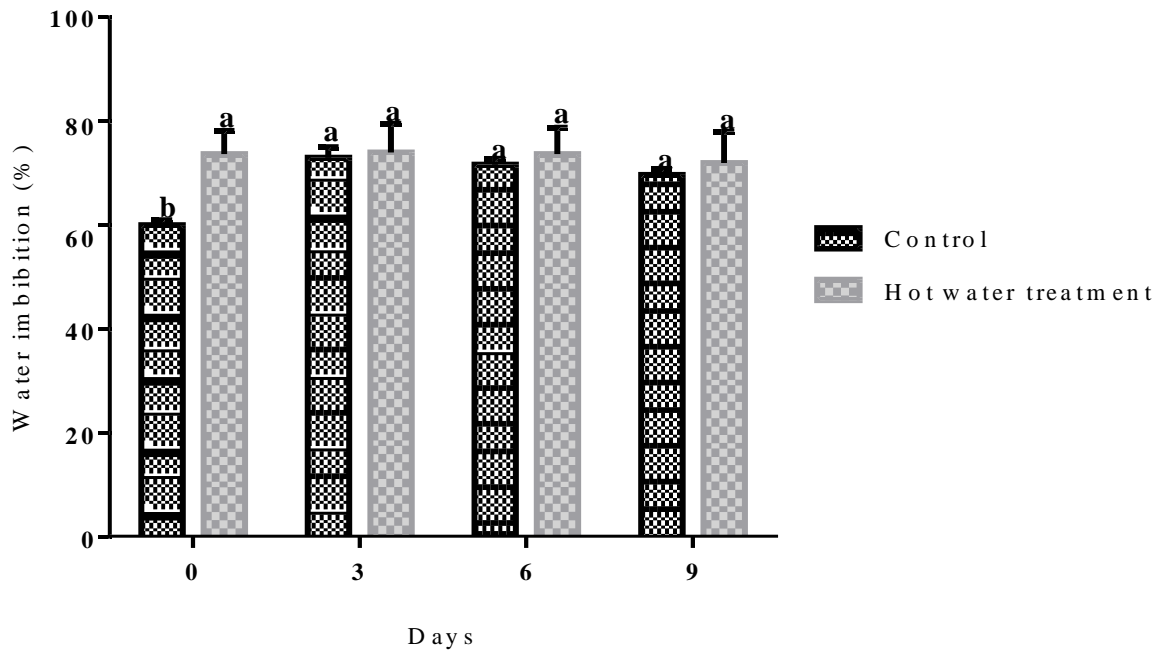
Figure 6: Water imbibition by seeds of *R. rubiginosa*. Bars with different letters are significantly different at 95% confidence interval using Turkey's test.

4.4   Sterilization Protocol

Contamination of explants was monitored for 15 days and the first contamination was recorded on the fourth day of culture. RG-MZ had the highest effect on eliminating contamination giving 73.0% followed by AT-325SC with 60.5% and finally AWP-70 with 54.3% of survival explants. The prevalent microbial contaminants were fungal with few incidence of bacterial contamination (Figure A, B).
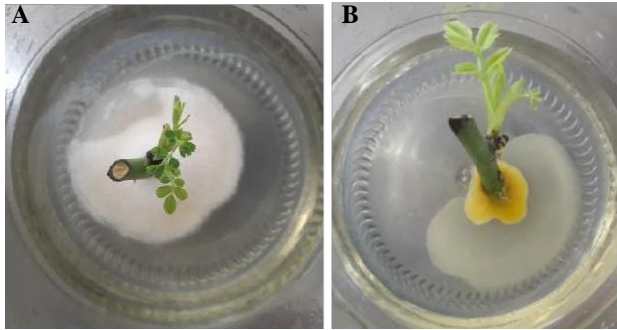
Figure 7: Sterilization protocol of *R. rubiginosa*. Explant contaminated with fungus (A) and bacteria (B) after 10 days of culture.

In a preliminary study, where the explants were treated for thirty minutes with 3 mg/ml ridomil, there was 100% fungal contamination. As such, for successive sterilization procedures, the chosen concentrations of the fungicides were higher than those recommended by the manufacturer for all the three fungicides.
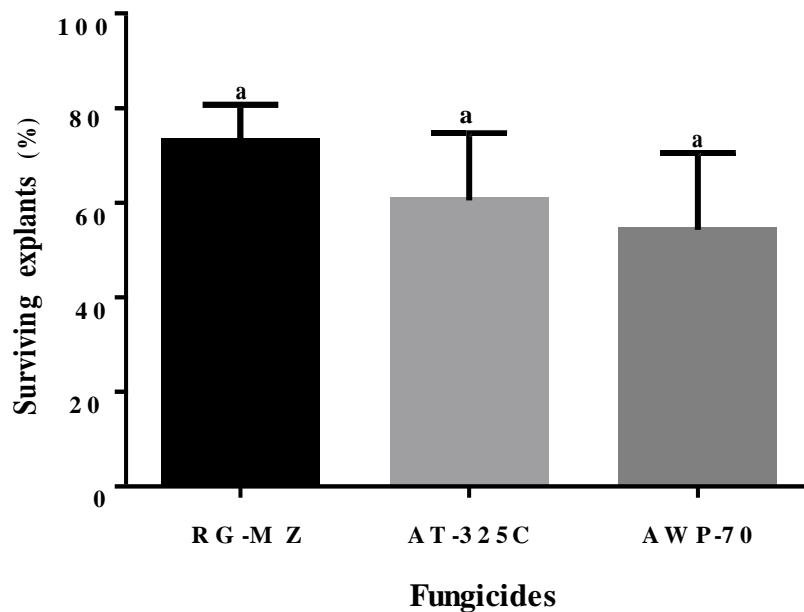
Figure 8: Effect of three fungicides on sterilization of nodal explants of *R. rubiginosa*. The bar graphs with different letters are significantly different at 95% confidence interval according Turkey's test.

4.5 Effect of Ascorbic Acid, Activated Charcoal and BAP on Shoot Induction

In the present study the effect of ascorbic acid, activated charcoal and BAP on shoot induction of from nodal explants of *R. rubiginosa* was investigated. The shoot induction of 100% was recorded on shoot induction medium containing 0.5 mg$\cdot$L^{-1} ascorbic acid, 0.5 g$\cdot$L^{-1} activated charcoal or 2.0 mg$\cdot$L^{-1} BAP. It was observed that at concentrations beyond 0.5 mg.L-1 (ascorbic acid), 500 mg.L-1 (activated charcoal) or 2.0 mg.L-1 BAP the shoot response was inhibited.
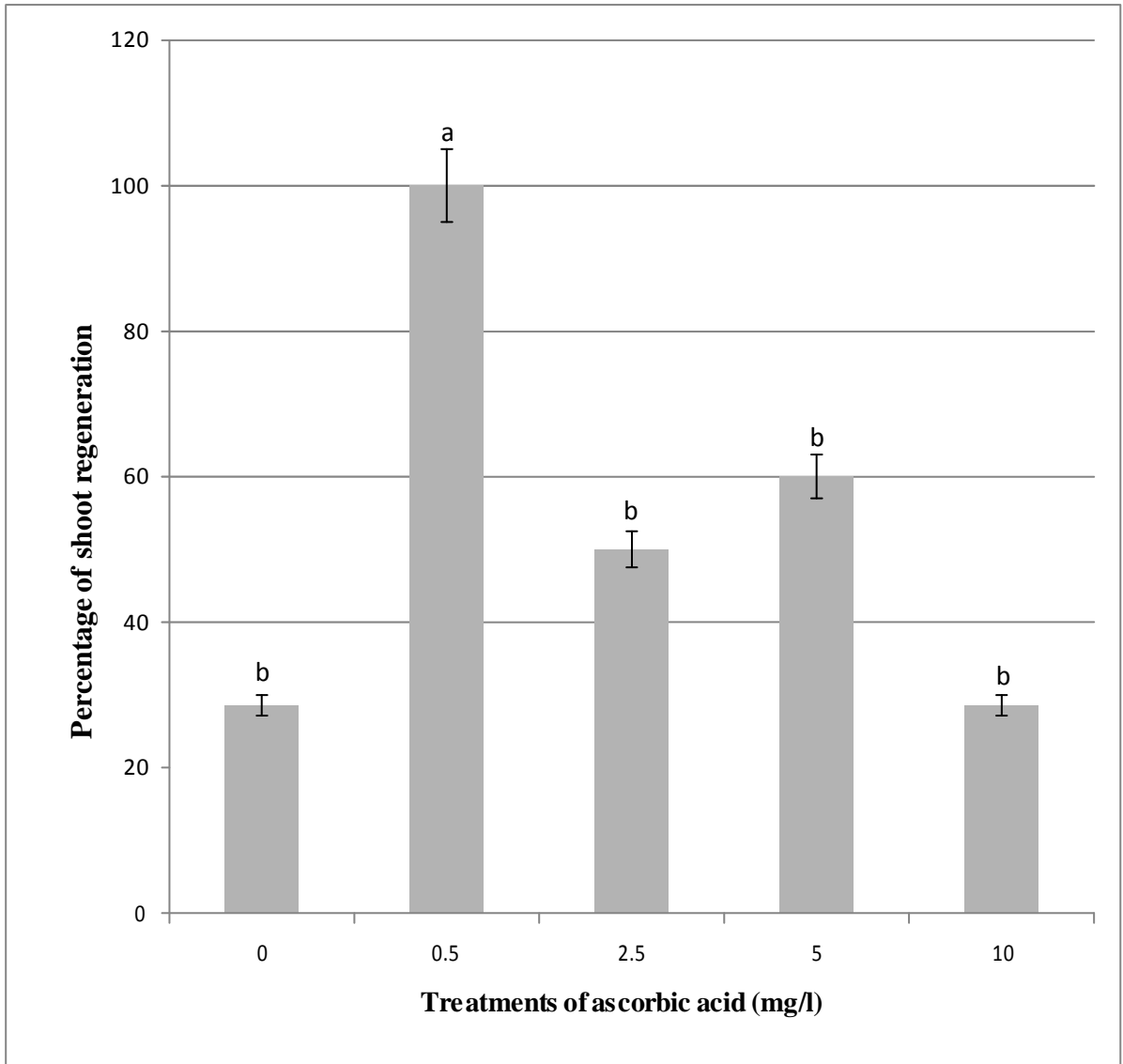
Figure 9: Shooting response on MS medium with various treatments of ascorbic acid. Treatments with different letters are significantly different at 95% confidence interval according to Turkeys test.
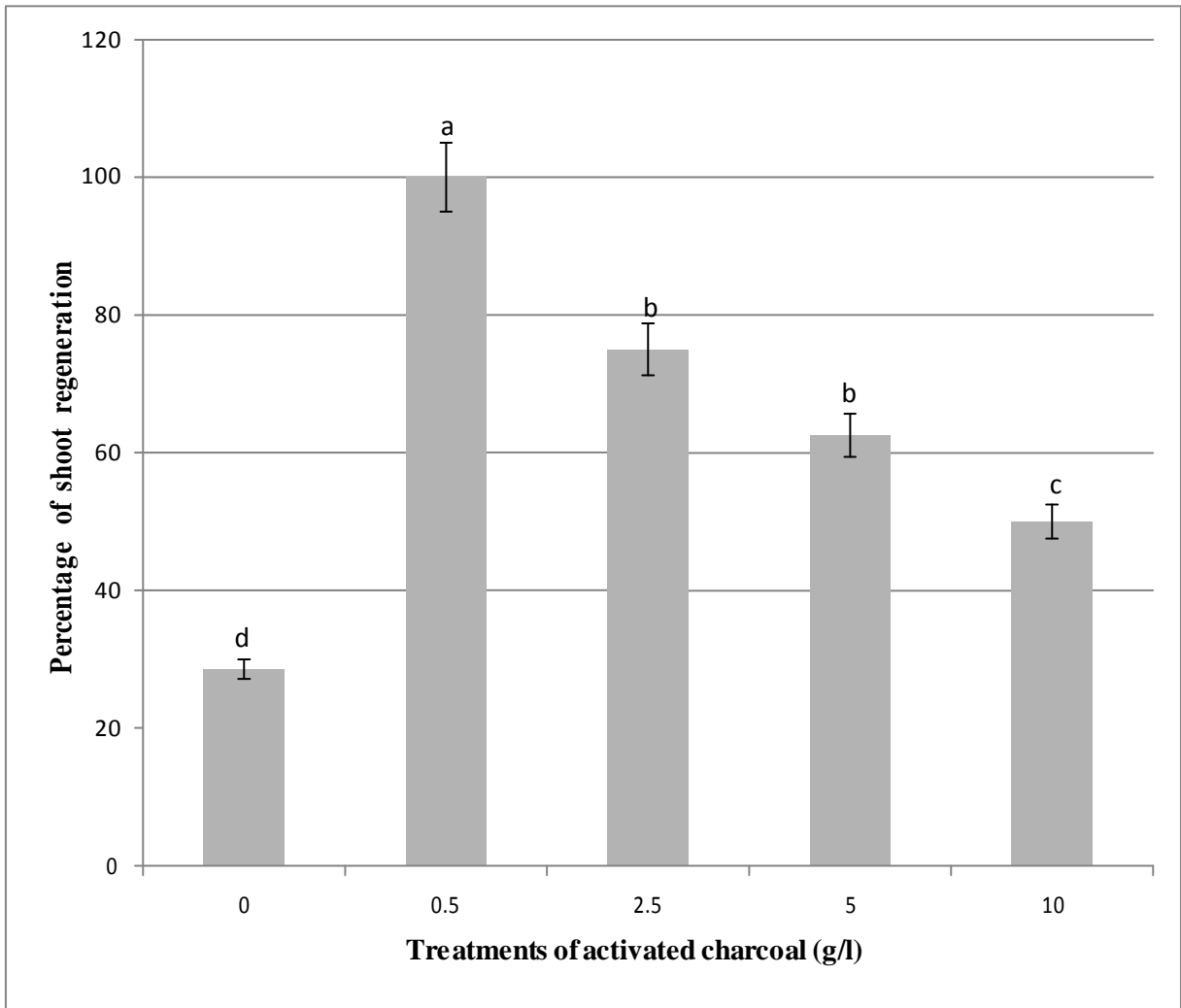
Figure 10: Shooting response on MS medium with various treatments of activated charcoal. Treatments with different letters are significantly different at 95% confidence interval according to Turkeys test.
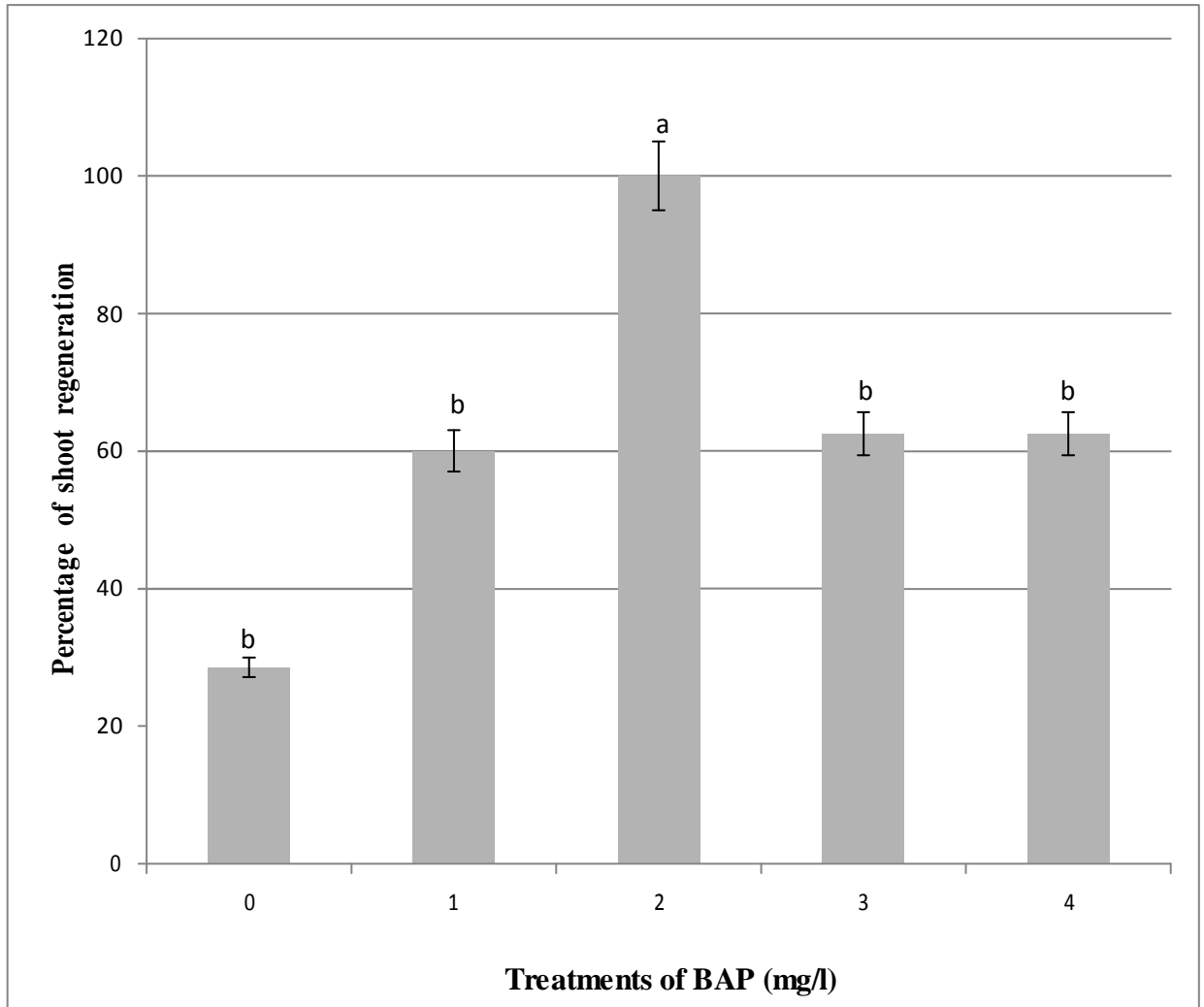
Figure 11: Shooting response on MS medium with various treatments of BAP. Treatments with different letters are significantly different at 95% confidence interval according to Turkeys test.

Following four weeks of culture, the number of shoots per explant was recorded. The highest number of shoots per explant was recorded on MS medium with 2.0 mg·L^{-1} BAP, followed by 0.5 g·L^{-1} AC and then 0.5 mg·L^{-1} AA (Table 5).

Table 5: Average number of induced shoots and their corresponding lengths

Treatment	Concentration (mg/l)	Average No. of shoots per explant	Length (cm)
	0.0	0.3^{b}	1.5^{ab}
	0.5	1.0^{a}	0.88^{ab}
Ascorbic Acid	2.5	0.5^{ab}	0.55^{b}
	5.0	0.4^{ab}	3.0^{a}
	10.0	0.5^{ab}	1.5^{ab}
	0.0	0.3^{b}	1.5^{a}
	0.5	1.1^{a}	0.8^{abc}
Activated Charcoal	2.5	0.4^{b}	0.9^{ab}
	5.0	0.8^{ab}	0.15^{c}
	10.0	0.8^{ab}	0.43^{bc}
	0.0	0.3^{b}	1.5^{a}
	1.0	1.0^{ab}	0.82^{a}
BAP	2.0	1.4^{a}	0.94^{a}
	3.0	0.8^{ab}	1.4^{a}
	4.0	0.8^{ab}	1.0^{a}

The values with different letters within the same column for each treatment are significantly different according to Turkey's test at 95% confidence interval.

4.5   Effect of Explants Orientation on Shoot Induction

There was no significant difference in response of nodal explants cultured vertically or horizontally (Table 6). In both orientations, direct shoot regeneration was observed. The sign for shoot formation was observed on the fifth day of culture characterized by swelling of the nodal explants (Figure 12).

Table 6: Effect of explant orientation on shoot induction

BAP Concentration (mg/l)	Shoot regeneration (%)	
	Vertical	Horizontal
0.0	33[c]	33[c]
1.0	100[a]	100[a]
2.0	67[b]	67[b]
3.0	100[a]	100[a]
4.0	33[c]	0[d]

Percentages with the same superscript letters are not significantly different as determined by Turkey's test at 95% confidence interval.
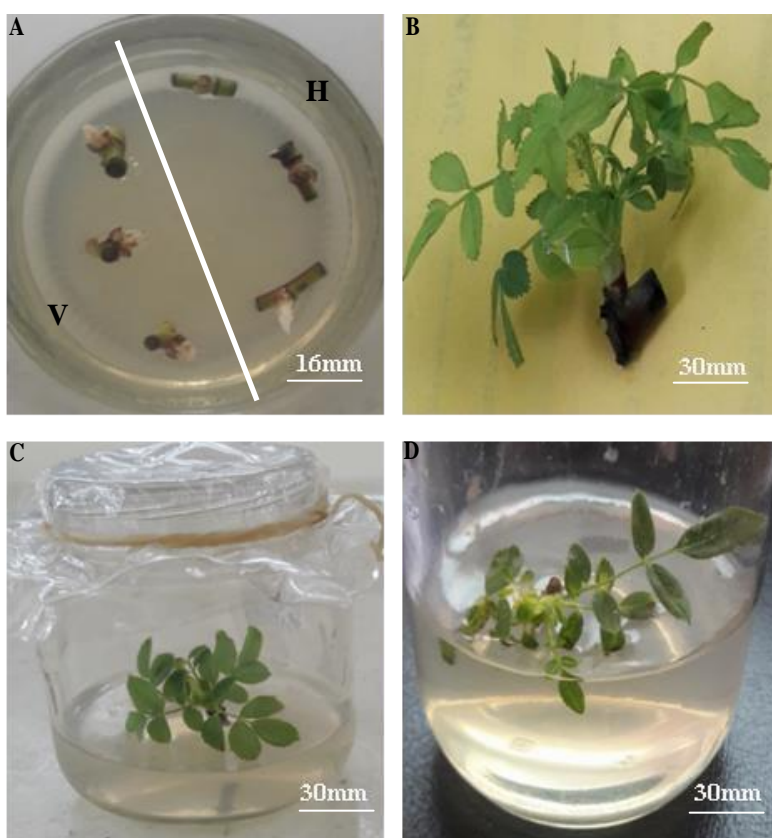
Figure 12: Nodal explants on MS medium with various concentrations of BAP. Response of nodal explants after 5 days of culture, 3 explants on V labeled section cultured vertically and in H section cultured horizontally (A), shoots on MS medium

with 1.0 mg·L^{-1} BAP on a horizontal orientation **(B)**, 1.0 mg·L^{-1} BAP on a vertical orientation **(C)** and without BAP **(D)** after 25 days of culture.

When the shoots were transferred to rooting medium, some shoots developed green callus at the base where they were in contact with the medium after a period of 3 weeks (Figure 13). The observed callus was compact and growing rapidly. However, other treatments did not show any signs of callus formation rather the explants showed signs of withering characterized with yellowing of leaves and eventually died.

Figure 13: Shoots on rooting treatments. Shoot on MS medium with 5.0 mg·L^{-1} NAA (A), shoot on MS medium with 0.5 mg·L^{-1} IAA (B), shoot on MS medium with 0.5 mg·L^{-1} IAA plus 0.5 g·L^{-1} activated charcoal (C) and shoots on MS medium with various treatments of IBA (D).

4.6   Callus, Rhizoid and Shoot Induction

The first sign of callus initiation was observed in the course of the second week of culture with more vigorous callus formation occurring in the fourth week of culture (Table 7).

Table 7: The effect of 2,4-D on callus formation

Treatment $(mg·L^{-1})$	Total number of explants	Callus formation (%)	
		After 4 weeks	After 8 weeks
0.0	15	13.3^d	13.3^c
1.0	15	46.7^{bc}	93.3^a
1.5	15	80.0^a	86.7^a
2.0	15	33.3^{cd}	66.7^b
2.5	15	60.0^{ab}	80.0^{ab}

Percentages with different superscript letters within the same column are significantly different at $p \leq 0.05$ using Turkey's test.

The white callus was observed along the edge of the leaf explants towards the point where it was attached to the petiole (Figure 14B). However, some explants formed callus at the edges even though it was not as vigorous as at the point where it was attached to the petiole. More rapid callus formation was observed on medium containing 1.0 mg·L^{-1} than on medium with other treatments. Following subculture, the callus became friable and light brown in color (Figure 14C).
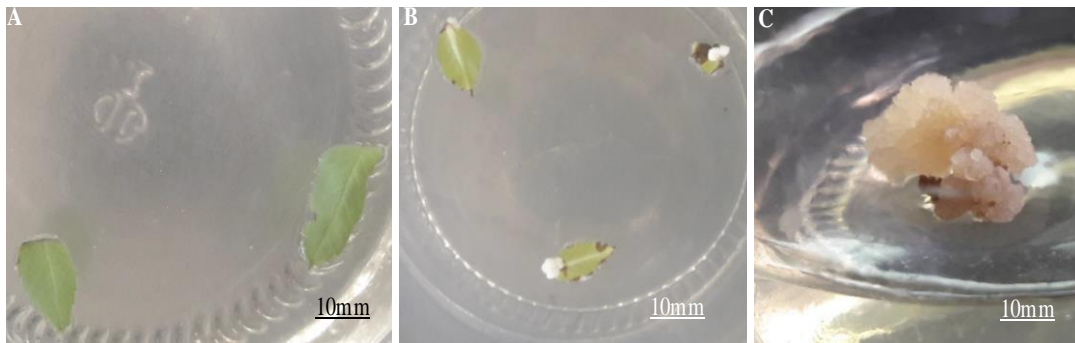
Figure 14: Effect of 2,4-D on callus induction from leaf explants of *R. rubiginosa*. Initiation of callus on MS medium with 1.0 mg·L^{-1} (A), callus formation on MS medium with 1.0 mg·L^{-1} callus after 4 weeks of culture (B) followed by callus on MS medium with 1.0 mg·L^{-1} 2,4-D after 4 weeks subculture (C).

Callus was sub-cultured on various treatments of 2,4-D and rhizoid formation was obtained on a treatment of MS medium with 2.0 mg·L^{-1} 2,4-D in darkness after a period of 4 weeks. No rhizoid formation was observed on other treatments (Figure 15).
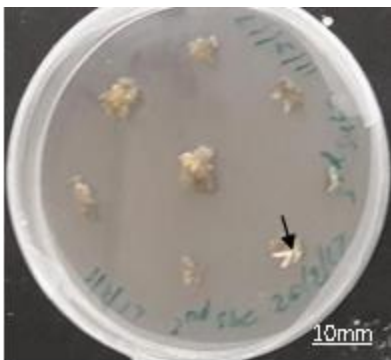
Figure 15: Formation of rhizoids on MS media with 2.0 mg·L^{-1} 2,4-D. Callus with rhizoid forming shown with an arrow.

Table 8: Effect of 2,4-D on rhizoid induction from callus

2,4-D Concentration (mg·L^{-1})	Number of explants	Rhizoid formation (%)
0.0	9	0^b
0.5	9	56^a
1.5	9	0^b
3.0	9	0^b
6.0	9	0^b

Percentage values with different letters are significantly different according to Turkey's test at 95% confidence interval.

For shoot induction, after first subculture on MS medium with 1.0 mg·L^{-1} 2,4-D, sub-cultured callus was transferred to MS medium with various treatments of BAP and NAA as shown in table 3. The cultures were incubated in the presence of light and the callus turned green and showed some rapid increase in size without formation of shoots after 4 weeks of culture (Figure 16).

Figure 16: Green callus formation on shoot induction medium after 4 weeks in the presence of light on MS medium supplemented with 0.1 BAP + 0.5 NAA mg·L^{-1}.

CHAPTER FIVE: DISCUSSION

5.0   General Discussion

The possible failure of germination from the seeds may not be related to one possible factor since there may be many complex inherent factors that regulate germination of dormant seeds. However, it could be that seeds pretreated with hot water had high concentration of ABA which may have been highly expressed due to change in temperature during hot water treatment. According to Toh *et al.*, (2008), the expression of ABA in imbibed seeds increases at high temperatures. Moreover, some seeds were cut longitudinally and many had shriveled embryos and this could be another possible failure of germination for both pretreated and untreated seeds used for germination test.

Sulphuric acid is known for disrupting seed coat which results in exposure of the lumens of the macrosclereids cells thereby permitting imbibition of water, which activate germination. Jackson (1994) reported that as soon as the hard pericarp of a seed was softened by scarification, hydrolysis commenced resulting to release of simple sugars that could be readily utilized in protein synthesis, thereby encouraging germination.

However, in this study, scarification of the seeds with sulphuric acid or with combination of sulphuric acid with hot water treatment was not effective to break seed dormancy of *R. rubiginosa*. These results are in agreement with that of Haouala *et al.*, (2013), who reported that pretreatment of seeds with hot water treatment at 97°C for 24 hours or with concentrated sulphuric acid at different durations (30, 45, 60 minutes) did not break seed dormancy of *R. rubiginosa*.

In another study, Nadeem *et al.*, (2013), firstly placed the seeds at warm conditions (25°C) for 30 days followed by transfer to cold conditions (4°C) for 60 days. Following completion of warm- and cold-stratification, the pretreated seeds were acid scarified with 30% sulphuric acid for 10 minutes to break seed dormancy of *Rosa* x *hybrida*. These treatments resulted in a germination percentage of 18.54.

Probably the concentration of ABA in the seeds reduced during the warm- and cold-stratification. Hence combination of acid scarification with warm- and cold-stratification was effective in inducing germination since both physiological and physical dormancy factors were targeted. It has been proved that during cold stratification, the expression of ABA reduced in the seeds (Chien *et al.*, 1998). Therefore, it is apparent that one of the major factors to consider during seed dormancy studies is to identify the time period at which the concentration of ABA in the reduced to a level where it cannot arrest germination.

Water is among the major requirements for seed germination because of its significant roles in seed physiology including activation of the enzymes, breakdown, translocation, and use of stored material in the seed. Thus, dormant seeds are also known to be metabolically inactive due to low water content. Therefore, in this study, imbibition test was carried out to determine if hot water treatment improves the imbibition properties of *R. rubiginosa* seeds.

It is a fundamental requirement for a seed to imbibe enough water in order to activate the germination process. The sufficient amount of water needed for activation of germination in a seed is referred to as critical moisture content and so every seed has its own specific critical moisture content. The seeds with known critical moisture content include soybean

(49.3%), Chinese cabbage (44.0%), hot pepper (42.9%), pea (41.5%), sesame (39.4%), radish (35.0%), barley (34.0%), corn (32.1%), wheat (32.0%) and rice (29.5%) (McDonald *et al.*, 1994).

During the imbibition studies, the moisture content of the seeds was recorded as 28%. However, there is no information on the critical moisture content optimum for activation of germination in the seeds of *R. rubiginosa*. Therefore, it is possible that the amount of imbibed water was not adequate to activate germination or perhaps other factors such as the ABA content played a role in the failure of the seeds to germinate.

For dye penetration test, there were no traces of dye stains inside the seeds when they were cut open. The fact that there were no traces of the dye observed when the seeds were soaked for 24 hours in 1% congo red and thereafter cut longitudinally implied that the dye was not able to penetrate the seed coat. Based on this observation, it could be concluded that hot water treatment was not effective enough to improve the imbibition properties of *R. rubiginosa*.

The seeds of *R. rubiginosa* have a thick, hard pericarp that inhibits germination (Haouala *et al.*, 2013) by physically restricting the development of the embryo (Nasr *et al.*, 2013). Techniques such as scarification with hot water and acid treatment are known to reduce the toughness of the seed coat (Schmidt, 2000). Therefore it could be that the duration of exposure of the seeds to hot water treatment was not sufficient to weaken the hard seed coat of *R. rubiginosa* seeds or alternatively, hot water treatment alone is not effective.

Failure of dye penetration into the seeds could further prove that hot water treatment did

not improve permeability of the hard pericarp of the seeds. As a result, the physically imposed dormancy due to hardness of the seed coat might have also played a major role in inhibiting seed germination. Various pretreatment techniques have been published for reducing seed dormancy and hasten germination, however, no single technique has been found to be equally effective for all seed species (Amusa, 2011).

Different seeds have different capacities for water uptake and the rate of water uptake is affected by various factors including seed composition, permeability of the seed coat as well as the availability of water (McDonald *et al.,* 1994). The seeds with high protein content have higher water absorption capacity than those with high starch content. According to McDonald *et al*., (1994), proteins possess both negative and positive charges a property that increases their affinity to polar charged water molecules thus they imbibe more water with easy.

Besides the toughness of the pericarp, seeds of *R. rubiginosa* also have high amounts of polysaccharides and oil content and lower amounts of protein (Dourato *et al.,* 2000). Seeds rich in polysaccharides have lower water affinity as a result they only absorb a minimal amount of water. The composition of the seed coat of *R. rubiginosa* could also play a role in water penetration into the seed although it was not investigated seed coats are made of cellulose and hemicellulose.

In this study, sterilization protocol of stem segments with a single node from naturally grown *R. rubiginosa* L. has been developed using antiseptic (savlon), disinfectants (NaOCl and ethanol), and fungicides (RG-MZ, AWP-70 and AT-325C). Fungal contamination is among the major enemies in tissue culture of *Rosa* species, they may be introduced by contaminated explants, airborne contaminated culture environment or

improper handling of explants during the experiment (Sen *et al.*, 2013).

Antibiotics, fungicides, heat and light are normally used in tissue culture to overcome both fungal and bacterial contamination (Leifert *et al.*, 1992). Alvarenga *et al.*, (2002), used RG-MZ as surface sterilant for micro-cuttings of cacao plant and observed that a treatment of 1 gL^{-1} RG-MZ increased browning of the explants was not effective since 100% fungal contamination was obtained. In addition, Sen *et al.*, (2013) used RG-MZ at various concentrations of 0.5 to 5% in combination with mercuric chloride at 0.1% for surface sterilization of nodal explants of *Achyranthes aspera* L. It was observed concentrations below 3% of RG-MZ were toxic as it resulted in browning of the explants (Sen *et al.*, 2013).

In this study, the explants were sourced directly from their natural habitat in the wild and many researchers have had difficulties in obtaining 100% clean explants from wild grown plants when used directly as source of explants (Odutayo *et al.*, 2007; Rout *et al.*, 1999). In the present study, 100% fungal contamination was observed when explants were treated with 3 gL^{-1} RG-MZ for 30 minutes.

According to the manufacturer, RG-MZ contains Metalaxyl-M as a systemic active compound and Mancozeb which forms a protective layer on the surfaces of the treated plants where it inhibits fungal spores' formation. Interestingly, when duration of treatment of explants was extended to 60 minutes and RG-MZ concentration increased to 20 gL^{-1} fungal contaminants were significantly reduced resulting in 73% uncontaminated explants. In contrast to the observation by Sen *et al.*, (2013), in the present study, no browning or shoot inhibition was observed on the explants treated with high concentration of RG-MZ.

Ascorbic acid (AA), activated charcoal (AC) and 6-benzylaminopurine (BAP) were used at varying concentrations to investigate their effect on shoot induction. AA is commonly used in tissue culture to inhibit oxidation of phenolic compounds that are released from the cut ends of the explants which when oxidized causes the medium to become toxic to the explants (Ahmad *et al.*, 2013).However, the principle behind inhibition effect on shoot response by AA has been reported. Chekizie (2012) studied the effect of AA inclusion ranging from 2.5 mgL^{-1} upwards during the regeneration of *Musa* species. It was reported that inclusion of AA in the tissue culture medium inhibited sprouting of the shoot tips of *Musa* species.

In the present study, AA was used at concentrations ranging from 0.5 to 10 mgL^{-1} and a 100% shoot induction was observed on 0.5 mgL^{-1} AA. The concentrations beyond 0.5 mg/l starting from 2.5 mgL^{-1} partially inhibited shoot induction of *R. rubiginosa*. AA is known for its rapid degradation due to oxidation in the medium especially when the medium is incubated in the presence of light. Therefore different authors argue that the effect of AA may only be observed within 3 days of culture after which it may no longer be present in the medium.

According to Chikezie (2012), AA is rapidly oxidized to dehydroascorbic acid and it is this subsequent compound that is correlated with inhibition of the shoot development. The principle behind mode of action for dehydroascorbic acid is that it deters the activity of various enzymes such as dehydrogenase, fructose 1,6- biphosphate and hexokinase which are necessary for the development of the explants *in vitro*.

Therefore based on the results of the present study, it may be possible that at lower concentrations only a small amount of dehydroascorbic acid is formed which does not

have significant effect on shoot induction of *R. rubiginosa*. However, as the concentration of ascorbic acid increases, significant amounts of dehydroascorbic acid accumulate in the medium thereby affecting the activity of the forementioned enzymes and so inhibiting shoot induction from the explants.

The effect of AC on shoot induction during tissue culture has been studied by several researchers. Among the major roles of AC during tissue culture is its capacity to adsorb inhibitory substances from the medium. AC is characterized by several pores that adsorb large number of particles in the medium (Thomas, 2008). Constantin *et al.*, (1977) reported that AC inhibited shoot development of tobacco by adsorbing the hormones required by the shoots for their development.

In addition, Chikezie (2012) reported that AC may even absorb the nutrients such as vitamins from the medium thereby resulting in inhibitory effect of the shoot development. However, if incorporated at appropriate amounts, AC may promote shoot development in tissue culture medium by adsorbing inhibitory substances such as phenolic exudates that cause browning of the medium which subsequently cause the medium to be autotoxic to the explants (Thomas, 2008).

In the present study, the inhibitory response observed at concentrations beyond 0.5 $g L^{-1}$ AC may be attributed to its high adsorption activity including adsorption of medium nutrients such as vitamins required by the explants. As result the medium became insufficient to support the shoot development of the explants. At lower concentrations however, activated charcoal may be adsorbing minimal amounts of vitamins but adsorbing good amounts of the inhibitory substances such as phenolic exudates thereby favoring increased shoot induction from the explants.

Moreover, in the present study, shoot development was also induced on MS medium containing various treatments of BAP. It has been reported by Khosh-Khui and Sink, (1982) that different genotypes of roses respond differently in different media and culture conditions. This was also reported in a study conducted by Bressan *et al.*, (1982), who found that BAP at concentrations ranging from 0.13 to 1.3 µM gave optimum proliferation.

In the present study, BAP was effective to induce shoot formation without combination with another plant growth hormone. However, Moallem *et al.*, (2012), reported that BAP alone was not effective to induce significant shooting in *R. canina*. Optimum shoot induction of 100% was only observed when BAP was combined with gibberellic acid (GA3) resulting in a fastest shoot induction within 7 days of culture.

There are many studies on the regeneration of roses that have been published so far (Moallem *et al.*, 2013; Davoudi *et al.*, 2015; Ambros *et al.*, 2016) though they are dedicated on other *Rosa* species other than *R. rubiginosa*. However, *Rosa* species are known for high heterogeneity which makes it difficult to apply a single protocol for regeneration of all *Rosa* species (Khosh-Khui, 2014). As such, the results on regeneration of roses show diverse responses even under the same culture condition.

Zapata *et al.*, (1999) also reported that growth regulators can be omitted in shoot induction medium for roses because their meristems have the ability to initiate shoots on their own. Therefore the results obtained in this study are in agreement with the findings of Zapata *et al.*, (1999) since 100% shoots were successfully induced on medium lacking growth hormones. However, despite this observation, the highest number of shoots per explant (1.4± 0.3) was recorded on medium supplemented with 2 mg·L^{-1} BAP.

In this study, the stem segments with single axillary buds were used as the starting material for the regeneration of *R. rubiginosa*. The major advantage of this kind of explant is that it gives stable plantlets which are more fertile (Barve *et al*., 1984) than the plantlets derived from the adventitious shoots which are less stable (Skirvin *et al*., 1990). The effect of explant orientation on shoot induction was also investigated in the present study.

However, there was no significant difference observed between the explants cultured in either vertical or horizontal orientation. This is different from the findings of a study conducted by Sharma *et al*., (2010), working with *Murraya koenigii* (L.) plant species who reported that nodal explants of *Murraya koenigii* (L.) cultured vertically had a higher shoot induction than those cultured horizontally.

Furthermore, Sharma *et al*., (2010) observed that in horizontally cultured explants, there was excessive excretion of phenolic compounds which might have killed the explants. Moreover, Sharma and the group therefore, proposed that vertically cultured explants had a proper acropetal channeling of nutrients by vascular tissues thereby accelerating their shoot induction than horizontally cultured explants.

Various treatments of NAA, IAA and IBA were prepared as shown in table 3. However, all the experiments failed to induce rooting from the shoots of *R. rubiginosa*. Commonly used auxins for rooting of roses include NAA, IBA and IAA. In this study, all these hormones were used separately and in combination yet they did not induce root development.

According to the literature, there are several factors that affect the rooting capacity of roses including light, genotype of explant source and temperature as well as salt

concentration of the nutrient medium (Douglas *et al.*, 1989). It is reported that majority of rose plants respond well to rooting when cultured on MS medium containing half or quarter strength of salt concentrations which can even eliminate the requirement for addition of auxins in the medium (Skirvin *et al.*, 1990).

In the present study however, the shoots failed to root when cultured on full, half and quarter strength MS salts both in the presence and absence of auxins. Arnold *et al.* (1995) reported that as the concentration of salts increase, the amount of IBA and NAA required for optimum root growth was also increase in *R. kordesii* cv. Champlain. Moreover, it was observed that when an auxin was added to the media the average length of the roots reduced in all cultivars under the study (Arnold *et al.*, 1995). Thus based on this hypothesis, the concentrations of NAA, IBA and IAA as high as 10 mg·L^{-1} in full strength MS medium was used yet no root induction was observed.

Another factor that affects rooting of roses is light intensity. According to Khosh-Khui and Sink (1982), shoots that were cultured under low light intensity (1.0 Klux) showed higher rooting response of 84% in comparison to those cultured at higher light intensities (3.0 Klux). However, in the present study, the light intensity in the culture room was about 2.0 − 2.2 Klux (40 - 44 μmol m^{-2}s^{-1}). For regeneration of *R. canina*, Tian *et al.*, (2008) successfully induced rooting on half-strength MS containing 0.2 mg·L^{-1} NAA when culturing the shoots of *R. canina* under 16/8h light/dark photoperiod with a light intensity of 120 μmol m^{-2}s^{-1}. In addition, Skirvin *et al.* (1990) reported that red light had positive effect on rooting of miniature roses (*R. chinensis*).

It was also reported that *R. chinensis* showed improved rooting response when cultured under cool white fluorescent light than under warm white fluorescent. In the present study,

the shoots of *R. rubiginosa* failed to induce rooting even when cultured in half-strength MS containing 0.2 mg.L^{-1} at 25$^{\text{o}}$C temperature and white fluorescent light provided by fluorescent bulbs. It is possible that the effect of light in combination with the temperature might have affected the response of these shoots.

Callus initiation was done on MS medium supplemented with various concentration 2,4-D using leaf explants. All the treatments showed white callus growth though more vigorous callus formation was observed on MS medium with 1.0 mg.L^{-1} 2,4-D. Jala (2014), successfully induced green callus from Miniature rose stem nodes with axillary buds when cultured on the medium containing 2,4-D in darkness. In contrast, in this study leaf explants from *R. rubiginosa* were used and white callus was obtained when cultured on the medium containing 2,4-D in darkness. This variation may be due to many possible factors including the difference in the type of explant used as well as the difference in the genotypes.

Rhizoid formation from callus was induced on MS medium supplemented with different levels of 2,4-D. Rhizoid formation was observed after four weeks of culture on MS medium supplemented with 3.0 mg.L^{-1} 2,4-D. A protocol for regeneration of *R. canina* through protocorm-like bodies that were induced from rhizoids was developed by Tian *et al.*, 2008 who observed that high rhizoid formation was obtained on the medium containing 1.5 mg.L^{-1} 2,4-D.

Rhizoids are white root-like structures that are induced by exogenous application of plant growth regulators such as auxins and inhibited by application of cytokinins (Gao *et al.*, 2013). Rhizoid formation has been reported in tissue culture of different plant species by different researchers including *R. canina, R. multiflora* var. *cathayensis* and *R. multiflora*

f. *carnea* (Tian *et al*., 2008; Gao *et al*., 2013).

Different shoot induction trials according to Safdari and Kazemitabar, (2010) and Jala, (2014) were used. However, no shoot formation was obtained from all of the trials attempted. Even though in this study *R. rubiginosa* was being used which is different from Mose Rose (*Portulaca grandiflora* L.) used in the study of Safdari and Kazemitabar (2010) both plants are under *Rosaceae* family.

Hence, failure to get shoots indicates that despite being in the same *Rosaceae* family, different *Rosa* species have different requirements for organ regeneration or organogenesis. Jala (2014) successfully induced shoots from callus of miniature rose when cultured on MS medium containing a combination of 1.0 mg·L^{-1} NAA with 1.0 mg·L^{-1} BA in the presence of light. In the present study however, a combination of NAA with BAP at different levels to induce shoots from callus failed to induce shoot formation.

It appears the culture conditions in the present study could not initiate cell fate changes that could lead to shoot formation. In order to form shoots from callus, the cells or callus undergo reprogramming to switch from just being cells to start forming specialized cells that eventually lead to shoot or organ formation. It appears the culture conditions could neither trigger cell fate changes nor reprogramming callus cells.

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Hot water and sulphuric acid treatments plus warm and cold stratification techniques did not break seed dormancy of *R. rubiginosa*. Moreover, hot water treatment did not improve water imbibition capacity of the seeds of *R. rubiginosa*.

Shoot induction from nodal explants of *R. rubiginosa* can be directly induced on MS medium without addition of plant growth hormone regulators. However, addition of plant growth regulators increases shoot induction as 100% shoot induction was obtained on MS medium treatment supplemented with 2.0 mg$\cdot$L^{-1} BAP. Moreover, treatments containing ascorbic acid and activated charcoal at 0.5 mg$\cdot$L^{-1} and 0.5 g$\cdot$L^{-1} respectively also gave 100% shoot induction. Culturing the nodal explants in either vertical or horizontal orientation on shoot induction medium did not show any significant difference.

6.2 Recommendations

Hot water treatment in combination with sulphuric acid scarification plus warm and cold stratification techniques did not break seed dormancy of *R. rubiginosa*. An alternative approach for breaking seed dormancy of *R. rubiginosa* would be through gene silencing of *abscisic acid insensitive-3* gene which is known to regulate expression of abscisic acid in the seeds of *Rosa* species.

Shoot induction protocol was successful thus the present protocol can be adopted for shoot induction of *R. rubiginosa*. However, different approaches such rooting in dark conditions and rooting at different temperature conditions as well as at different light sources such as

red light, white cool light and white warm light should be explored. In addition, various hormone combinations which were not tested in the present study should be tried.

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