DECLARATION

I, **Tashinga D Mudzengi**, do hereby declare that this thesis submitted by me is my own original work undertaken by myself except where clearly specified. I undertake that any quotation or paraphrase from published or unpublished work of another person has been duly acknowledged. This work has not been submitted for the fulfilment of any other degree or programme in any other university.

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Correct citation: Mudzengi TD (2014). Germination performance and β-1.3-glucanase expression of tobacco (*Nicotiana tabacum* L.) seeds to after-ripening temperature, dry heat treatment and imbibition solutions MSc. Thesis. Midlands State University.

DEDICATION

This dissertation is dedicated to my beloved mother it was your wish and through your sacrifices that I obtain the best education. Thank you, may the Lord be with you and richly bless you. Love you my mother.

ACKNOWLEDGEMENTS

I give glory and honour to God from whom everything is attained even the success of this project.

Mr T. Madanzi and Dr V. Makuvaro thank you for assisting me in making this dissertation a success and you were a great supervisors that anyone could think of and l could not have had better supervisors like you. Your assistance, devotion, and guidance throughout this work provided me with a strong, working knowledge base and many opportunities to grow and develop as a person as well as a professionally.

I acknowledge the financial support of Tobacco Research Board that allowed me the opportunity for graduate study involving the seed science hat has been such an important part of my life. I also extend special thanks to Dr. D. Rukuni for providing counsel and excellent facilities for conducting this research. A special thanks goes to Stan Banana for his assistance with data analysis. Special acknowledgement is extended to Fungai Mukange and Mike Marunda for their friendship in addition to their assistance, throughout my entire experience at TRB whose walking breaks and friendship kept me going in the toughest times.

I thank fellow graduate students, Justin Mbiza, Chirisa Chamisa, Wadzanai Viriri and Tendai Shiri for moral support. Words simply cannot say how much I appreciated the assistance and support of my brother his devotion to my research and me aided me in completing my master's degree I extend a special appreciation to my family for they remained supportive of me in the toughest times of my life and instilled a work ethic in me along with a passion and deep respect for seed science and agriculture in general that has helped me get to where I am today.

ABSTRACT

Erratic seed germination is one of the major challenges affecting tobacco production and adoption of improved varieties. This problem can be attributed to seed dormancy and environmental conditions. After-ripening is one of the pragmatic approaches used to enhance the germination of freshly harvested tobacco seeds however the process of after-ripening is a slow and there is need to explore other approaches such as imbibition in phyto-hormones (gibberellic acid and benzyl adenine), imbibition in hydrogen peroxide and dry heat treatment. The aim of the study was to determine the effect of after-ripening temperature, dry heat treatment and imbibition solutions on germination performance and β-1.3-glucanase expression of two selected new tobacco varieties *viz* KRK 26 R and T71. In the first experiment the effect of after-ripening temperature and imbibition solution effect on tobacco seed germination performance under four different germination environments (20 ^{o}C light, 20 ^{o}C dark, 30 ^{o}C light and 30 ^{o}C dark) was evaluated. This first experiment was a 2 x 4 factorial experiment in a completely randomised design with after-ripening temperature at two levels (5 ^{o}C and 30 ^{o}C) and imbibition solution in four levels (benzyl adenine, distilled water, gibberellic acid and hydrogen peroxide). Freshly harvested seeds were sampled from the two after-ripening temperatures every two weeks for eight weeks and imbibed in the four respective imbibition solutions. In the second experiment the effect of after-ripening temperature and imbibition solution on β-1, 3-glucanase activity in T71 and KRK 26 R incubated under 30 ^{o}C light and 30 ^{o}C dark was determined using the similar design as the first experiment. The enzyme activity of seeds stored for eight weeks at the two respective after ripening temperatures and imbibed in the four solutions was determined using the reducing sugar spectrophotometry method. The objective of the third experiment was to determine the effect of dry heat treatment (DHT) and imbibition solution on tobacco seed germination percentage performance under 30 ^{o}C light and 30 ^{o}C dark. This third experiment was a 4 x 4 factorial experiment laid out in completely randomised design with DHT done at 37 ^{o}C. The DHT had four levels (30 ^{o}C after-ripened seed + no DHT, 5 ^{o}C after-ripened seed + no DHT, 30 ^{o}C after-ripened seed + seven days DHT and 5 ^{o}C after-ripened seed + seven days DHT) and imbibition solution had four levels (benzyl adenine, distilled water, gibberellic acid and hydrogen peroxide). In the first experiment after-ripening temperature and imbibition solutions had interaction ($P < 0.05$) in all the varieties across all the weeks after storage (WAS) except for T71 (at 6 and 8 WAS) and KRK 26 R (2 and 4 WAS) seeds incubated at 20 ^{o}C dark. Under 20 ^{o}C light seeds after-ripened at 30 ^{o}C and imbibed in either hydrogen peroxide or water attained the highest germination rate. However using phyto-hormones did not significant improve germination rate except gibberellic acid for T71 seeds at 2 and 8 WAS. The first experiment also showed that gibberellic acid significantly improves germination percentage of the two varieties incubated in 20 ^{o}C dark, 30 ^{o}C light and 30 ^{o}C dark. Under similar conditions seeds after-ripened at 30 ^{o}C marginally improves germination regardless of the imbibition solutions. An interaction was noted between imbibition solution and after-ripening in the second experiment ($P < 0.05$) except for 30 ^{o}C dark incubated T71 seeds ($P > 0.05$). The second experiment showed that β-1,3–glucanase activity is enhanced by exogenous application of gibberellic acid under both 30 ^{o}C light and 30 ^{o}C dark. The exposure seeds at 30 ^{o}C after-ripening temperature marginally enhanced β-1,3–glucanase activity under the similar conditions. An interaction was attested between DHT and imbibition solution on germination percentage of seed of the two varieties incubated at 30 ^{o}C light and 30 ^{o}C dark in the third experiment ($P<0.05$). In the third experiment it was established that that DHT enhances germination percentage of 30 ^{o}C light and 30 ^{o}C dark incubated seeds.

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LIST OF ACRONYMS AND ABBREVIATIONS

ABA	Abscisic acid
ABRE	ABA-responsive elements
ANOVA	Analysis of Variance
AtCPS1	*Ent*-copalyl diphosphate synthase 1
BA	6-benzylaminopurine / benzlyadenine
bHLH	Basic helix-loop helix
BME3	Blue Micropylar End 3
CV	Coefficient of variation
DHT	Dry heat treatment
GA	Gibberellic acid
ISTA	International Seed Testing Association
LSD	Least Significant Difference
MD	Morphological dormancy
MPD	Morphophysiological dormancy
PD	Physiological dormancy
Pfr-form	Phytochrome far red form
PIL5	Phytochrome-Interacting-Factor-Like5
PY	Physical dormancy

PY+PD	Combinational dormancy
ROS	Reactive oxygen species
SED	Standard Error Difference of means
SPT	Spatula
TRB	Tobacco Research Board
USD$	United States Dollars
WAS	Weeks after storage
βGlu I	Class I β-1,3-glucanase

CHAPTER ONE

1.0 INTRODUCTION

Tobacco production is a viable enterprise, and has the potential to contribute towards improving incomes and livelihoods especially of rural small scale farmers in Zimbabwe (Masvongo *et al.*, 2013). Seed germination is a decisive step towards realizing economic success in seed transplant operations in tobacco with synchronized germination being important in attaining optimal crop stand and improved productivity (Wahid *et al.,* 2008). Consequently, the use of high quality seed enables achievement of uniform crop stands in the field and uniform seedling development which is desirable for cultural mechanized operations such as clipping (Mukarati *et al.,* 2014). Erratic seed germination is one of the main challenges faced in tobacco seedling production especially for new improved varieties (Kuboja *et al.,* 2011) leading to many farmers failing to realize their tobacco production potential.

Uniformity in germination and seedling emergence is critical in efficient float tray tobacco seedling production. Inconsistent seedling emergence, such as a 2-day delay in seed germination results in tobacco seedlings that are small thus reducing overall seedling usability and even if the seedling is transplanted after 50 days it still will shows reduced growth. (Cundiff *et al.,* 1978; Hartely *et al.,* 2002). The interaction effect of the environment and seed physiological factors controls the germination process (Edelstein and Nerson, 2005) and are responsible for erratic germination in tobacco seeds. Fresh seeds have non-deep physiological dormancy, which means the embryo has low growth potential such that it cannot overcome physical constraint exerted by the testa and the micropylar endosperm (Baskin and Baskin, 2004). This hampers the production of maximum and uniform transplants in float seedling production system during crop establishment (Wang *et al.,* 2007). Seed dormancy leads to the delay of germination in any specified period under any combination of normal physical environmental factors that are

otherwise favorable for tobacco seed germination (Baskin and Baskin, 2004; Silveira, 2011). Consequently it is imperative to break seed dormancy to facilitate seed germination (Tavakkol *et al.,* 2011).

Apart from dormancy, synchronized germination is also impeded by environmental conditions (Wahid *et al.,* 2008) with the prevailing temperature conditions being cardinal in tobacco seed germination (Kurt, 2010). Germination of non-dormant tobacco seeds occurs over a wide range of temperatures while of germination of dormant seeds occurring in a very narrow environmental window (Baskin and Baskin, 2004). Specifically germination of tobacco seeds is best under a temperature range of range 18 to 23 ^{o}C (Hartely *et al.*, 2001). Lower temperatures lead to poor and slow germination while higher temperatures cause thermo-inhibition (Ghassemi-Golezanik *et al.,* 2008) and thermo dormancy. Tobacco seeds are also positively photoblastic hence they require light to activate the phytochrome system and initiate germination (Finkelstein *et al.,* 2008) and incubation in the dark reduces seed germination (Leubner-Metzger *et al.,* 2001).One pragmatic technique used to enhance and stabilize field emergence, which is the basis of crop success, is seed after-ripening.

Non-deep physiological dormancy in tobacco can be alleviated by after-ripening (Leubner-Metzger and Meins, 2000; Probert, 2000; Kucera *et al.,* 2005; Bair *et al.,* 2006). Prolonging dry storage (after-ripening) increases the germination ability of seeds and widens the environmental window thus allowing germination to occur in a wider range of environmental conditions (Bazin *et al.,* 2011). Environmental temperature is of paramount importance to the success and rate of after-ripening (Probert, 2000; Finch-Savage and Leubner-Metzger, 2006; Holdsworth *et al.,* 2008) and but generally increased temperature are associated with increased after-ripening (Bazin *et al.,* 2011).

However, after-ripening is a slow process and additional approaches can be used to facilitate germination such as imbibition in phyto-hormones such as 6-benzylaminopurine/ benzly adenine (BA) and gibberellic acid (GA) as well as hydrogen peroxide (H_2O_2). Leubner-Metzger *et al.* (1996); Finch-Savage and Leubner-Metzger, (2006); Mukarati *et al.* 2014 reported that exogenous application of GA can alleviate dormancy in freshly harvested seeds and can allow germination to take place in dark. BA a common cytokinin, has been found to promote germination in *Lactuca sativa* seeds (Cantilife 1991; Nascimento and Cantlife, 1998) and in combination with moist chilling, in tobacco seeds (Mukarati *et al.,* 2014).

Hydrogen peroxide (H_2O_2) has been found to break dormancy and facilitate germination of various crops through two mechanisms; first by the scission and loosening of cell wall polymers which is imperative for cell extension of embryos during seed germination (Muller *et al.,* 2007). Exogenous application of hydrogen peroxide has been used in various crop plants such as *Malus domestica* (Bogatek *et al.,* 2003), *Arabidopsis thaliana* (Liu *et al.*, 2010), *Hordeum vulgare* (Fontaine *et al.,* 1994; Wang *et al.,* 1998; Cavusoglu and Kabar, 2010), *Oryza sativa L* (Naredo *et al.,* 1998) and *Zinnia elegans* (Ogawa and Iwabuchi, 2001). Dry heat treatment has been used to promote germination of *Elaeis guineensis* (Chanprasert *et al.,* 2012), *Erica australis* (Vera *et al.,* 2010) and in *Gossypium hirsutum* (Basra *et al.,* 2004). Mukarati *et al. (*2014) reported that there is a marked improvement in germination of primed tobacco seed after incubation at 40 ^{o}C for seven days.

The micropylar endosperm offer the greatest physical constraint to radicle protrusion during tobacco seed germination thus it has to be weakened to facilitate germination (Leubner-Metzger, 2005). Endosperm weakening is influenced by the abscisic acid (ABA) and GA balance as well as hydrolases such as mannose, cellulose, and glucanase (El-Maarouf-Bouteau and Baily, 2008). Of

importance in tobacco is the enzyme β-1, 3-glucanase which facilitates endosperm weakening and subsequent radicle protrusion (Vogeli-Lange *et al.*, 1994; Leubner-Metzger *et al.*, 1995; Leubner-Metzger, 2005). The knowledge of activity of β-1, 3-glucanase in tobacco seeds can help in explaining the germination trends.

Since tobacco seeds germinate poorly, the cultivation of tobacco can be enhanced and increased production can be attained by facilitating germination in the fields. Information on the combined effect of storage after-ripening temperature, dry heat treatment and imbibition solutions on germination performance is important in understanding where the inefficiencies in tobacco seedlings production lie so as to explore strategies which can be used to improve germination especially for new varieties such as KRK 26R and T71 released by Kutsaga Seeds, Zimbabwe. The research also was aimed at exploring the effects of after-ripening temperature and imbibition solutions on β-1,3-glucanase expression in tobacco seeds.

1.1 Overall objective

To explore the effect of after-ripening temperature, dry heat treatment and imbibition solutions on germination performance and β-1.3-glucanase activity of two selected new tobacco varieties *viz* KRK 26 R and T71.

1.1.1 Specific objectives.

1. To evaluate effects of after-ripening temperature and imbibition solutions on tobacco seed germination performance under four different germination environments *viz* 20 ^{o}C light, 20 ^{o}C dark, 30 ^{o}C light and 30 ^{o}C dark.

2. To determine the effects of after-ripening storage temperature and imbibition solutions on β-1, 3-glucanase activity in tobacco seeds incubated at 30 ^{o}C in light and dark.

3. To determine the effects of dry heat treatment and imbibition solution on tobacco seed germination performance under 30 ^{o}C light and 30 ^{o}C dark.

1.2 Hypothesis

1. Germination performance of tobacco seed under the four different environmental conditions (20 ^{o}C light, 20 ^{o}C dark, 30 ^{o} light and 30 ^{o}C dark) varies with after-ripening temperature and imbibition solutions.

2. After-ripening temperature and imbibition solutions affect the activity of β-1, 3-glucanase in tobacco seed at 30 ^{o}C light and 30 ^{o}C dark

3. There is an effect of dry heat treatment and imbibition solutions on tobacco seed germination performance incubated under 30 ^{o}C light and 30 ^{o}C dark.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Tobacco production in Zimbabwe

Tobacco historically has been a crop of choice and vital export product for Zimbabwe as early as 1920s (Rukuni *et al.,* 2006) with production occupied predominantly by white settler farmers until 2000 (Kamuti *et al.,* 2013; Masvongo *et al.,* 2013). The introduction of the fast track land reform program in 2000, saw the transition in the tobacco agrarian structure with the commercialization of small-holder tobacco production (Kamuti *et al.,* 2013). For the past five years, tobacco production especially among small-holder farmers has been on the increase and subsequently increasing demand in inputs such as fertilizers and seeds (Masvongo *et al.,* 2013). By 2012, an increase in the number of registered farmers was noted, from 15 000 in 1998 to 64 775 in 2012 for which 80 % being smallholder farmers (Saunyama, 2013; Mangudhla, 2013). In 2012 Zimbabwe received USD$ 771 million from tobacco which contributed 40 % of the total exports and 10 % of the Gross Domestic Product (Kamuti *et al.,* 2013), this underlines important to the people of Zimbabwe.

Tobacco provides livelihoods for over one million Zimbabweans, who are either directly or indirectly employed by the industry (Kamuti *et al.,* 2013). With the ever increasing small-holder farmers there is now need the use of high quality seed since tobacco production is now being done across all regions. This signifies the importance of seeds research and an extension in literature which need to be explored.

2.2 The problem of low seed germination in tobacco.

Erratic seed germination have been noted among improved varieties consequently leading to low adoption of these varieties (Kuboja *et al.,* 2011) this can be attributed to physiological aspects and environmental conditions. The problem of low seed germination can be caused by factors such as seed size, stresses during production, nitrogen supply, harvesting time and storage facilities. The environmental effect can lead to differences in seed size, which impact tobacco seed germination. Tso (1990) demonstrated that heavier seeds germinates faster than lighter seeds so a seed lot with weight differences lead to unsynchronized tobacco seed germination.

Low and erratic tobacco seed germination can be caused by environmental stress such as nutrient deficiency, water stress, temperature stress and shading (Clarke, 2001). Increased supply of nitrogen during tobacco seed production is critical in promoting uniform germination. Thomas and Raper (1979) reported that increasing supply of nitrogen leads to increased germination and uniformity in tobacco seed germination. The timing of harvesting is one factor that contributes to low tobacco seed germination, Gwyn (1973) reported that seeds harvested 14 days after anthesis showed lower germination than seed that were collected 21 days after anthesis. However seed dormancy, temperature and light are the most important factors causing low germination in tobacco (Bunn and Splinter, 1961; Kasperbauer, 1968) this signifies the importance of research covering the three thematic areas.

2.3 Germination definition and process

Seed germination is fundamental step in tobacco crop production and has profound effects on production of high quality crop. From a seed physiologist view point, seed germination can be defined as all events that commence with water uptake by a seed and terminate with the elongation

of the embryonic axis (Bewley and Black, 1994). The visible sign of the completion of the germination process is normally the penetration of the structures surrounding the embryo by the radicle; the result is often called visible germination. From a seed analyst, technologist and farmer point of view germination is the emergence and development important structures which are indicative of the ability to produce a normal plant under favorable conditions. In other words these the three groups' measures germination only after a normal plant is observed. Cantliffe (2000) outlined that a crop producer counts seedlings that are emerged from the soil and only considers pullable seedlings. Germination is determined by seed structure, seed quality, seed dormancy, pre-treatment (release from dormancy) and germination conditions such as water, temperature, substrate, light, and freedom from pathogens.

2.4 Tobacco seed physiology and germination

Huthchens (1999) described a tobacco seed as possibly one of the smallest seed of all agricultural crops with a size of approximately ≤ 1 mm. Tobacco belongs to *Solanaceae* family, sub group *Ceistroidea* which possess endospermic seed, which characterized by an abundant endosperm layer that serves as a food storage organ. The embryos are straight or slightly bent and the seeds are prismatic to subglobose (Leubner-Metzger, 2003). The embryo of mature tobacco seeds are surrounded by three to five layers of thick walled endosperm cells and the periphery of the endosperm layer is pressed against the testa, which consists of an outer layer of cutinized and lignified dead cells and a living inner parenchyma layer. The maternal origin of this living cell layer is interposed between the endosperm and the dead outer testa interposed between the endosperm and the dead outer testa.

In tobacco the two covering tissues, the endosperm and testa are the mechanical barriers which must be overcome by the growth potential of the embryo if the seed is to complete its germination (Linkies *et al.,* 2009). The micropylar endosperm is the main tobacco seed germination constraining structure and consequently endosperm weakening by hydrolases is a perquisite for the completion of germination (Leubner-Metzger, 2003). Germination in tobacco is characterized by a two-step process *viz* testa rupture and endosperm rupture which are separate and sequential events (Krock *et al*., 2002; Petruzzelli *et al.,* 2003). The plant hormone ABA inhibits endosperm rupture and endosperm weakening, but not necessarily the testa rupture in tobacco seeds and its inhibitory effect of ABA is counteracted by GA (Kucera *et al*., 2005; Muller *et al.*, 2006).

2.5 Seed dormancy definition and its relationship with germination

Dormancy is defined as the inability or delay of a seed germination, under any combination of normal physical environmental factors that are favourable for germination of that species (Baskin and Baskin, 2004).Seed dormancy is under the influence of the combination of genetic factors and the environment. (Finch-Savage and Leubner-Metzger, 2006). Seed dormancy provides adaptation to a diversity of habitats therefore, it is an important component of plant fitness (Donohue *et al*., 2005; Huang *et al*., 2010).

Reduced dormancy is undesirable since it increase seed deterioration in storage consequently risking seedling mortality and increased seed dormancy levels delay germination thus lengthen the growing season (Donohue *et al*., 2010). However, a balanced dormancy level is desirable. When the farmer sow seeds, they should germinate immediately, making seed dormancy an unwanted trait (Debeaujon *et al.,* 2000).

Bethke *et al*. (2007) alluded to the fact that the endosperm plays pivotal role as a tobacco germination regulatory barrier. Germination and dormancy depend on the balance between the growth force of the elongating radicle and the resistance strength of the surrounding tissues. The activities of cell wall remodelling proteins influence the strength of the surrounding tissues (Leubner-Metzger 2005; Endo *et al*., 2012), whereas the force of the radicle is determined by elongation of cells in the transition zone and lower hypocotyl (Sliwinska *et al.,* 2009).

2.6 Seed dormancy classification

Seed dormancy can be distinguished as primary dormancy and secondary dormancy. Primary dormancy develops during seed maturation on the mother plant (Hilhorst *et al.,* 1998) while secondary dormancy is induced in seeds with non-deep physiological dormancy. Once primary dormancy, subsequently secondary dormancy is induced if the conditions required to stimulate germination are absent (Baskin and Baskin, 2004). Secondary dormancy can be lost and re-introduced repeatedly as seasons change until the required germination conditions become available.

Seed dormancy classification as proposed by Baskin and Baskin (1998, 2004) comprises a comprehensive classification system which includes five classes of seed dormancy: physiological (PD), morphological (MD), morphophysiological (MPD), physical (PY) and combinational (PY+PD). PD is the most abundant form dormancy class in the field and noted in crop species such as *Arabidopsis thaliana*, *Helianthus annuus*, *Lactuca sativa*, *Lycopersicon esculentum*, *Nicotiana tabacum* and *Avena fatua* (Finch-Savage and Leubner-Metzger 2006). Physiological dormancy is has three levels deep, intermediate and non-deep (Baskin and Baskin, 2004) and of importance is the non-deep level which is possessed by tobacco seeds.

Seed with deep physiological dormancy have embryo that produces abnormal seedling excised, exogenous GA does not promote germination and seeds may require 3 to 4 months of cold stratification to facilitate germination (Baskin and Baskin, 2004). In intermediate physiological dormancy include, excised embryo produces normal seedling and exogenous GA may promote germination in some of the species. These seeds require two to three months of cold stratification or after-ripening facilitates germination through dormancy breaking. (Baskin and Baskin, 2004). Tobacco seeds has non-deep physiological dormancy and the excised embryo produces normal seedlings and exogenous GA promotes germination (Koornneef *et al.*, 2002). Depending on species cold stratification or warm stratification, after-ripening and scarification may promote germination. Mechanical resistance because of combined effect of the testa and endosperm that is greater than the embryo growth potential is the cause of primary non-deep physiological dormancy in tobacco seeds (Hilhost, 1995; Bewley, 1997: Leubner- Metzger, 2003).

Morphological dormancy (MD) in seeds is characterised by embryos that are underdeveloped in terms of size, but differentiated meaning they have distinct structures such as cotyledons and hypocotyl. In addition the embryos are not physiologically dormant, but simply require time to grow and germinate (Baskin and Baskin, 2004; Finch-Savage and Leubner-Metzger, 2006). Example of species with morphological dormancy include; *Apium graveolens.* Morphophysiological dormancy (MPD) is also evident in seeds with underdeveloped embryos, but in addition they have a physiological component to their dormancy (Baskin and Baskin, 2004). These seeds therefore require a dormancy-breaking treatment, for example a defined combination of warm and/or cold stratification which in some cases can be replaced by GA application.

Physical dormancy (PY) is caused by water impermeable layers of palisade cells in the seed or fruit coat that control water movement. Mechanical or chemical scarification can break physical dormancy. Examples of species with physical dormancy include; *Melilotus* and *Trigonella* (Baskin and Baskin, 1998; Baskin, 2004). Combinational dormancy (PY + PD) is evident in seeds with water-impermeable coats as in physical dormancy, combined with physiological embryo dormancy (Baskin and Baskin, 2004). Examples are the winter annuals such as; *Geranium* and *Trifolium.*

2.7 Induction and maintenance of primary dormancy

ABA has been detected in all seed and a fruit tissue examined and has been related to a number of developmental processes, including synthesis of storage proteins and late embryogenesis-abundant proteins, suppression of precocious germination, and induction of desiccation tolerance (McCarty, 1995). ABA is an important positive regulator of both the induction of dormancy and the maintenance of the dormant state in imbibed seeds following shedding and attainment of desiccation tolerance (Hilhorst, 1995; Bewley, 1997; Li and Foley, 1997; Koornneef *et al.,* 2002; Kucera *et al.,* 2005; Finch-Savage and Leubner-Metzger, 2006).

In most of the species, ABA levels increase during the first half of seed development and decline during late maturation as the seed water content decline. Studies with ABA-deficient mutants of arabidopsis and tomato have demonstrated that defects in ABA synthesis during seed development result in the formation of non-dormant seeds (Groot and Karssen, 1992). Deficiency of ABA during seed development influences the absence of primary dormancy in the mature seed while over expression of ABA biosynthesis genes can increase seed ABA content consequently enhancing seed dormancy or delaying germination (Kushiro *et al.,* 2004; Finch-Savage and

Leubner-Metzger, 2006). High ABA contents is associated with strong dormancy and as the dormancy reduces ABA level also reduces (Ali-Rachedi *et al.,* 2004; Cadman *et al.,* 2006) providing evidence that ABA biosynthesis is associated with the dormant seed state.

Endogenous ABA, impose enduring dormancy whereas maternal ABA or ABA exogenous application has limited effect on inducing lasting dormancy. (Kucera *et al.,* 2005). Lefebvre *et al.* (2006) in their work concluded that ABA synthesised both in the embryo and in the endosperm both contribute to the induction of seed dormancy. Transcriptome analysis with *Arabdopsis thaliana ecotype* Cape Verde Island by Lefebvre *et al.* (2006) showed that increased ABA biosynthesis is associated with the dormant state. *De novo* ABA biosynthesis during imbibition of dormant seeds has been demonstrated in the *A. thaliana* ecotype Cvi (Ali-Rachedi *et al.,* 2004), *Nicotiana plumbaginifolia* (Grappin *et al.,* 2000), *Helianthus annuus* (Le Page-Degivry and Garello, 1992) and *Hordeum vulgare* (Wang *et al.,* 1995). This *de novo* ABA biosynthesis has been interpreted as a mechanism for dormancy maintenance.

Ali-Rachedi *et al.* (2004); Cadman *et al,* (2006) alluded to the fact that dormancy depends on an intrinsic balance of GA and ABA biosynthesis. Ali-Rachedi *et al.* (2004) suggested that in dormant seeds a feedback mechanism exists that maintains a high ABA: GA ratio. Thus, the net result of the dormant state is characterized by increased ABA biosynthesis and GA degradation. Karssen and Lacka (1986) concluded that, ABA and GA act at different times and sites with ABA inducing dormancy during maturation and GA playing a key role in dormancy release and germination promotion.

However evidence provided with *Sorghum bicolor* by Steinbach *et al.,* 1997 with ABA deficient and insensitive mutants of *Zea mays* (White and Rivin, 2000) demonstrated that GA and ABA can act at the same time on dormancy and germination. The ABA: GA ratio and not the absolute

hormone contents control germination is responsible influence dormancy and germination. Thus, it seems that GA directly antagonizes ABA signalling during dormancy induction of cereal grains. This provides a knowledge gap therefore an extension in literature with other species are needed to determine whether this is a general phenomenon.

Dormancy maintenance depends on high ABA: GA ratios and dormancy release involves a net shift to increased GA biosynthesis and ABA degradation resulting in low ABA: GA ratios (Cadman *et al.,* 2006). This buttress the work of Le Page-Degivry *et al.* (1996) that ABA is the primary hormone involved during dormancy maintenance and release and that GA should be in sufficient concentrations to promote germination onces ABA biosynthesis is inhibited. In *Avena fatua* (Fennimore and Foley, 1998) it is reported that exogenous GA stimulates germination of but not through embryo dormancy loss but through in stimulating embryo growth. Thus, dormancy release is characterized by the capacity for enhanced ABA degradation and increased GA biosynthesis, which is followed by GA promotion of seed germination.

Nakabayashi *et al*, (2005) made an important finding that the dormant state is characterized by the transcription of genes with an over representation of ABA-responsive elements (ABRE) in their promoters and of genes for transcription factors that bind to the ABRE. The over representation of ABRE-containing genes which is evident in stored mRNAs of dry orthodox seeds with the ABRE-binding transcription factors are responsible for mediating ABA responses in seeds and regulation of dormancy. GA-responsive genes are also produced during imbibition but GA also causes down-regulation of many ABRE-containing genes (Yamaguchi and Kamiya, 2002; Yamauchi *et al.,* 2004).

Bioactive GAs accumulate in the embryo en route for radicle protrusion with light inducing GA biosynthesis, which occurs in two separate embryo tissues during germination: (1) the provascular

tissue, where *ent*-copalyl diphosphate synthase 1 (*AtCPS1*) gene promoter activity is localized, has the early biosynthetic pathway, including the geranylgeranyl diphosphate cyclization reaction catalysed by CPS; (2) the cortex and endodermis of the root, where GA 3-oxidase 1 (*AtGA3ox1*) and *AtGA3ox2* transcripts accumulate and *AtGA3ox2* gene promoter activity is localized, have the late biosynthetic pathway, including the formation of bioactive GA by GA3ox. This physical separation of the early and late GA biosynthetic pathway implies that intercellular transport of an intermediate (probably *ent*-kaurene) is required for the production of bioactive GA by the embryo.

Yamaguchi and Kamiya, 2002; Yamauchi *et al,* (2004) and Penfield *et al,* (2005) concluded that cold and light responses are mediated through the promotion GA biosynthesis via enhanced expression of *AtGA3ox*. The Blue Micropylar End 3 (BME3) GATA zinc finger transcription factor which is expressed in the radicle is involved as a positive regulator of seed germination and GA biosynthesis in response to cold stratification (Liu *et al.,* 2005). Penfield *et al,* (2005) explains that the control by cold and light through the interaction of the basic helix-loop helix (bHLH) transcription factors Spatula (SPT) and Phytochrome-Interacting-Factor-Like5 (PIL5). SPT and PIL5 are both active as repressors of germination in dark while in light plus cold their repressive activities are low. The regulation of PIL5 activity is controlled at the level of protein stability by light, which causes its repressive activity to decrease. In dark stratified seeds, SPT activity appears to be dependent on PIL5 (Liu *et al.,* 2005).

Cadman *et al,* (2006) suggests that there is active biosynthesis of GA precursors in dormant and non-dormant seeds with AtGA20ox1 transcripts always present at high abundance leading to the conclusion that biologically inactive GA 9 and GA 20 are always produced. There is also a high abundance of *AtGA2ox1* transcripts present in all states and any biologically active GA formed are degraded rapidly. In seeds that require only light to germinate when exposed to light, the transcript expression of *AtGA3ox2* increases dramatically, presumably completing the final step of the

biosynthesis of biologically active GA. Therefore, a dynamic balance of biosynthesis and degradation of ABA and GA may exist that determines a state-specific equilibrium in the ABA: GA ratio (Cadman *et al.,* 2006). High ABA signalling is associated with dormancy and high GA signalling with germination, while the transition between the two programmes is controlled by shifting the signalling between the two hormones (Liu *et al.,* 2005).

Conclusions regarding the role of ABA and GA concentrations or synthesis in dormancy and germination are valid for the regulation of embryo dormancy. However, the emerging depictions are incomplete without considering coat dormancy and hormone sensitivities. The sensitivities for GA and ABA, their perception by receptors, their interconnected signalling chains, and their developmental regulation are of utmost importance for germination and dormancy (Kucera *et al.,* 2005). In addition to hormone content and synthesis, the transition from the dormant to the non-dormant state of many seeds is characterized by a decrease in ABA sensitivity and an increase in GA sensitivity (Le Page-Degivry *et al.,* 1996; Corbineau *et al.,* 2002; Leubner- Metzger, 2002; Chiwocha *et al.,* 2005).

Besides genetic factors, the environment has a profound influence on the acquisition of dormancy during seed development. So far, no generalizations have been made as to which environmental factors result in consistent effects on dormancy over a range of species. Diverse effects of a variety of environmental factors on the development of seed dormancy have been reported, including day length, light quality, mineral nutrition, competition and temperature, physiological age of plants and position of seeds on the parent plant.

2.8 Role of light on tobacco seeds germination

It is widely accepted that temperature regulates both dormancy and germination and that light regulates germination; however, it is a matter of debate whether light is also a regulator of dormancy or a germination requirement (Baskin and Baskin, 2004; Kucera *et al.*, 2005). Light has been considered both as a stimulant of germination or terminator of dormancy (Batlla and Benech-Arnold, 2004). Exposure of tobacco seeds to red light facilitates germination in dark and is therefore the last step in the dormancy-breaking process, rather than the first step in the germination process (Leubner-Metzger, 2003).

Tobacco seed is positive photoblastic seeds meaning its germination is either stimulated or inhibited by light. In tobacco seeds with coat dormancy, light can releases dormancy and promote germination (Leubner-Metzger and Meins, 2000; Sanchez and Mella, 2004; Kucera *et al.*, 2005). This light effect (red light via phytochrome) can also be reversed in some cases by far-red light, until the seed is committed to the process of germination (Sanchez and Mella, 2004).

The phytochrome system in tobacco seeds, consists of two types of blue-green pigments; the phytochrome red form (Pr-form) and phytochrome far red form (Pfr-form). The germination promoting pathway involves absorption of the red light (660 nm) by the Pr-form which is then converted into the Pfr-form (Oh *et al.*, 2006). In presence of the far red light / dark the Pfr form absorbs far red light (730 nm) and is converted back into the Pr form thereby inhibiting seed germination, however the Pfr is slowly converted to Pr (Oh *et al.*, 2006). The net transformation from the inactive Pr form to the active Pfr form during the course of a changing photoperiod affects seed germination (Sanchez and Mella, 2004) and despite the effect of light quality, light intensity is a major factor governing the rate of germination. In seeds the amount or quantity of photons

received by an imbibed seed is important in eliciting a phytochrome response (Leubner-Metzger, 2003).

The induction of germination by red light can be substituted by the application of GA (Leubner-Metzger, 2003). Red light application does not induce germination in mutants deficient in GA (Oh *et al.,* 2006). Presence of red light stimulates the GA biosynthetic gene's expression encoding *GA2ox (LsGA2ox2)* in lettuce and *AtGA3ox1* and *AtGA3ox2* in Arabidopsis, whereas far red light inhibits the GA biosynthetic gene's expression encoding GA3ox (Yamauchi *et al.,* 2007). During tobacco seed germination it is not clear which gene expression is influenced by the red light this creates in extension in literature which need to be extended. Transcripts of a GA-deactivating genes *GA2ox* (*LsGA2ox2*) in lettuce and *AtGA2ox2* in Arabidopsis are reduced by red light (Yamauchi *et al.,* 2007; Oh *et al.,* 2006; ; Seo *et al.,* 2006). The ABA biosynthetic and deactivating enzymes are also regulated by light with genes encoding for ABA biosynthetic enzymes the NCED (*LsNCED2* and *LsNCED4* in lettuce and the Arabidopsis *AtNCED6* and *AtNCED9*) and zeaxanthin epoxidase (AtZEP/AtABA1 in Arabidopsis) are reduced by presence of red light (Sawada *et al.,* 2008) whereas, transcript levels of ABA-deactivating genes encoding CYP707A (*LsABA8ox4* in lettuce and CYP707A2 in Arabidopsis) are elevated by red light (Sawada *et al.,* 2008). The phytochromes regulate the levels of ABA and GA by one of the interrelating proteins

2.9 Effects of temperature on tobacco seed germination

Temperature is one of the factors that limit tobacco seed germination and germination occurs over a wide range of temperatures, with the optimum temperature for tobacco falling within the range of 18-23 ^{o}C (Hartley *et al.,* 2001). Though temperature influence is cultivar dependent, lower temperatures may delay emergence thereby increasing non-uniformity, while higher temperatures

may actually decrease germination (Huthchens, 1999). Temperature has a more pronounced effect on germination probability than either soil moisture or wavelength of light (Clarke, 2001).

Sub optimal temperatures delay and slow down germination process thereby delay emergence and causes non uniformity. Under supra-optimal temperature germination is reduced due to thermo inhibition and subsequent thermo dormancy through increased endogenous ABA production and reduced ethylene production.

2.10 Dry after-ripening effect on seed germination

Freshly harvest seeds may show some germination, particularly at low temperatures, but such seeds germinate slowly and non-uniformly. Consequently the seeds will eventually acquire the ability to germinate quickly and nearly completely after dry storage (Probert, 2000). After-ripening, a period of usually several months of dry storage temperature of freshly harvested, mature seeds, is a common method used to release dormancy (Bewley, 1997; Probert, 2000; Leubner-Metzger, 2003; Kucera *et al.,* 2005; Bair *et al.,* 2006).

After-ripening is characterised by results in a widening of the temperature range for germination, a decrease in ABA concentration and sensitivity and an increase in GA sensitivity or loss of GA requirement. It also led to loss of light requirement in seeds that do not germinate in darkness, an increase in seed sensitivity to light in seeds that do not germinate even with light and a loss of the requirement for nitrate. After-ripening increases germination rate of fresh seeds (Finch-Savage and Leubner-Metzger, 2006). Dry after-ripening is a dormancy-breaking process and occurs in the dry state with is rate highly temperature dependent (Bair *et al.,* 2006) but parameters such as moisture, oil content and seed-covering structures (Manz *et al.,* 2005) also influence seed after-ripening.

After-ripening is prevented in very dry seed (Manz *et al.*, 2005). The process require seed moisture content above the seed threshold value which is species-specific but lower in oilseeds compared to starchy seeds (Manz *et al.*, 2005). After-ripening is also prevented during storage at very high air humidity (Finch-Savage and Leubner-Metzger, 2006). Non-enzymatic reactions that remove germination inhibitors, reactive oxygen species and antioxidants (Bailly, 2004), specific protein degradation via the proteasome (Borghetti *et al.*, 2002) and membrane alterations (Hallett and Bewley, 2002) have been proposed as the molecular bases of after-ripening.

Bove *et al*. (2005) made available evidence that tobacco seed after-ripening generates a developmental switch at the transcript level which agrees with Cadman *et al*, (2006) on his work with A. *thaliana* Cvi transcriptome work. In *Avena fatua*, transcriptional regulation and post-transcriptional regulation are both important for the expression of dormancy-associated genes (Li and Foley, 1997). Bove *et al.* (2005) and Leubner-Metzger (2005) alluded to the fact that rapid promotion of testa rupture of tobacco seeds occurred after 60-80 days of dry storage (Leubner-Metzger, 2005). This was associated with transient β-1,3-glucanase gene expression in the covering layer during tobacco after-ripening. In addition Bove *et al*. (2005) found that at least eight specific mRNAs accumulated in air-dry, low-hydrated seeds of *Nicotiana plumbaginifolia* during after-ripening. Degradation of mRNAs and proteins for positive regulators of dormancy and for negative regulators of germination appears to be part of the molecular mechanisms of seed after-ripening, the possibility of *de novo* gene expression during seed after-ripening should also be considered.

2.11 Role of temperature in dry after-ripening

Seed responses to temperature plays a pivotal role in several germination syndromes and it is arguably the most important variable responsible for the synchronization of germination (Probert, 2000). Roberts (1988) outlined three seed physiological process influenced by temperature: firstly temperature in combination with moisture content decides the rate of deterioration in all seeds; secondly temperature affects the rate of the loss of dormancy in dry seeds and in moist seeds influences the pattern of dormancy change. Thirdly in non-dormant seeds temperature determines the rate of germination.

Temperature is a critical environmental factor that affects the status of dormancy during dry after-ripening storage. In most species, dormancy is alleviated faster with increasing storage temperature (Steadman, 2003) thus reducing the mean dormancy period. This has been evident in crops like barley (Favier and Woods, 1993), wild oat (Foley, 1994), cheat grass (Allen *et al.*, 1995) and annual rye grass (Steadman, 2004).

2.12 Role of GA in seed germination

Two functions of GA during seed germination have been proposed (Kucera *et al.*, 2005). Firstly, GA increases the growth potential of the embryo thus promoting germination. Secondly, GA is necessary to overcome the mechanical restraint conferred by the seed-covering layers by weakening of the tissues surrounding the radical through induction of the Class I β-1,3-glucanase and other hydrolases enzymes. GA promotes tobacco endosperm rupture by distinct signal transduction pathways. (Kucera *et al.*, 2005).

The β-1, 3- glucanase is induced after testa rupture and just prior to endosperm rupture of tobacco seeds (Leubner-Metzger, 2003 and Wu *et al.,* 2000). This induction is exclusively localized in the micropylar endosperm where the radicle will emerge. ABA inhibits the induction of βGlu I genes during tobacco seed germination and specifically delays endosperm rupture. Direct evidence for a causal role of βGlu I during endosperm rupture comes from sense-transformation of tobacco with a chimeric ABA-inducible βGlu I transgenic (Leubner-Metzger and Meins, 2000). The induction of cell wall hydrolases therefore promote endosperm weakening and subsequent endosperm rupture since the endosperm provides a mechanical barrier to the germination of seeds of tobacco. A decline in this mechanical resistance of the micropylar endosperm is a prerequisite for radicle protrusion during tobacco seed germination (Hilhorst, 1995; Bewley, 1997; Leubner-Metzger, 2003; Sanchez and Mella, 2004; Kucera *et al.,* 2005). This endosperm weakening can be promoted by GA and, at least in part, inhibited by ABA. Exogenous GA substitutes for the red-light trigger needed to release photo-dormancy thus inducing germination in dark (Kretsch *et al.,* 1995; Leubner-Metzger, 2003).

2. 13 β-1, 3-Glucanase causally contributes to endosperm rupture

In tobacco seeds, β-1, 3-glucanase (βGlu I) contributes to endosperm weakening thus promoting radicle protrusion. This can be either direct by degradation of ß-1, 3-glucan (callose) cell wall material, at the neck regions of plasmodesmata or as a callose layer directly below the testa. Or indirectly by the release of elicitor-active oligosaccharides that induce cell-wall degradation through the action of reactive oxygen species (ROS) that are known to cleave cell wall polysaccharides and cause cell-wall weakening.

βGlu I is transcriptionally induced in germinating tobacco seeds just prior to endosperm rupture, but after testa rupture. This induction is highly localized in the micropylar endosperm at the site of radicle emergence The induction of βGlu I and endosperm rupture are tightly linked in response to physiological factors known to affect the incidence and timing of germination such as light and GA (Leubner-Metzger, 2003).

2.14 Role of 6-Benzyilaminopurine / benzyl adenine in seed germination

Benzyilaminopurine or benzyl adenine (BA) is a form of Cytokinin and generally is able to break dormancy in the presence of gas or when the red irradiation is provided. Matilla (200), outlined that BA can only promote germination if they are exposed to a low level of red light indicating that minimum level of Pfr must be present. Many species are known where this cytokinin alone break seed dormancy (Matilla, 2000). During the conditioning of parasitic *Orobanche* and *Striga* species and the release of lettuce thermo inhibition, cytokinins appear to contribute to the promotion of dormancy release and subsequent germination by enhancing ethylene biosynthesis (Babiker *et al.*, 2000; Matilla, 2000).

A cytokinin–ethylene connection is also supported by the discovery that the Arabidopsis cytokinin-resistant1 mutant is insensitive to ethylene and allelic to the ethylene-insensitive mutant ein2 (Fischer-Iglesias and Neuhaus, 2001). Cytokinin-resistant mutants of *N. plumbaginifolia* have been isolated that exhibit reduced seed dormancy and pleiotropic seed effects suggestive of cytokinin–ABA interactions (Rousselin *et al.*, 1992).

2.15 Role of exogenous hydrogen peroxide in seed dormancy and germination

There is evidence that hydrogen peroxide (H_2O_2) alleviates seed dormancy. Exogenous H_2O_2 has been found to stimulate germination of dormant seeds of barley (Wang *et al.,* 1998; Fontaine *et al.,* 1994; Cavusoglu and Kabar, 2010), rice (Naredo *et al.*, 1998), apple (Bogatek *et al.,* 2003) zinnia (Ogawa and Iwabuchi, 2001) lettuce and pig weed (Hendricks and Taylorson,1975. In addition Oracz *et al*. (2007) demonstrated that cyanide, a compound releasing sunflower seed dormancy, triggered ROS accumulation and protein oxidation.

H_2O_2 seems to have a role in cellular response to ABA at the level of the gene expression and by regulating ion movement in guard cells (Oracz *et al*., 2007). Furthermore, in vitro biochemical studies revealed that H_2O_2 inactivates ABI1 and ABI2 type 2C protein phosphatase, enzymes that function in ABA signalling (Oracz *et al*., 2007). Treatment of dormant barley seeds with hydrogen peroxide resulted in a decrease in endogenous ABA level (Oracz *et al*., 2007) and alleviation of apple embryo dormancy by cyanide induced a simultaneous increase in H_2O_2 level and decrease in ABA content (Kolika *et al.,*2014). Kolika *et al.* (2014) concluded that the disproportionating of H_2O_2 results in an increased oxygen level, which enhance the oxidative respiration, is the rationale behind its effect on seed germination promotion.

Cano *et al.* (1997), in their research on *Lupinus albus* L. concluded that exogenous application of hydrogen peroxide can alleviate the effect of stress during seed germination, but has no effect on germination under normal conditions. In light of the effect of environmental stress and limited literature on the role of hydrogen peroxide, this study was aimed at providing additional literature on the effectiveness of hydrogen peroxide in enhancing tobacco seed germination.

2.16 Role of dry heat treatment in seed germination

In contrast to the immense literature on low temperature effects, there have been far fewer reports of the effect of warm temperatures on changes in germination behavior (Probert, 2000). In some species like *Spergula arvensis* (Karssen and Lacka 1985) and *A. thaliana* (Ali-Rachedi *et al.*, 2004) chilling and warming both release dormancy. In many other species, however, chilling and warming have opposing effects (Probert, 2000). In most summer annuals, low winter temperatures release dormancy, whereas high summer temperatures induce dormancy (Baskin and Baskin, 1987, Bouwmeester, 1990), while in winter annuals the reverse is true (Baskin and Baskin, 1986).

In a study of germination in desert perennial shrub species from South Africa, Gutterman (1990) demonstrated that the effect of pre-incubating seeds at 45 °C for 24 hours depended on whether plants originated from areas receiving winter or summer rain. In the former, germination was adversely affected, whereas, in the latter, germination was stimulated by high-temperature treatment. It is noteworthy that the promotive effect of dry heat treatment are often rapid, and there have been numerous reports of a positive interaction between high-temperature treatments and the control of dormancy by other factors, notably light (Probert, 2000). Taylorson and DiNola (1989) showed that the light requirement in *Echinochloa crus-galli* seeds was abolished in a significant proportion of individuals (37 %) following a 0.5 hour treatment of imbibed seeds at 46 °C. Accordingly, such a response could have ecological consequences for field emergence.

CHAPTER THREE

3.0 GENERAL MATERIALS AND METHODS

3.1 Site description

Kutsaga is located 15 km East of Harare along Airport Ring Road at geographical position 17^0 55`S; $31^0$ 08`E and altitude of 1479 m above sea level. The research station is in Natural Region II receiving an average of 750-1000 mm / annum (Vincent and Thomas, 1960). The mean average temperature during the seed production period was is 18 ^oC minimum and maximum 32 ^oC and rainfall 813 mm from data collected at the local weather station.

3.2 Seed material and variety description

Pure seed lots of Kutsaga flue cured tobacco varieties KRK 26 R and T71 used in this study were supplied by the Seed Production Division of Research Board (TRB), Kutsaga Research Station. All seed was produced during the 2013/2014 tobacco growing season and all the experiments were conducted as Kutsaga Seeds Laboratories.

Table 3.1. Description of KRK 26 R.

Variety	Description of the variety
KRK 26 R	Medium tall type of plant with fairly close internodes and yield potential of 4.0 t/ha. Fair uniformity and germination in seedbeds. Medium to fast ripening with uniform colouring and easy curing giving predominantly a lemon cured leaf. Establishes fairly well in the field. Lower reapings slightly prone to guinea fowl spot. Resistant to wild fire, white mould, angular leaf spot resistant, root knot nematodes, black shank and bacterial wilt and susceptible to alternaria.

Source: (TRB, 2012)

Table 3.2. Description of T71

Variety	Description of the variety
T71	Tall plant with close internodes, darker than KRK 26 R and suitable for all areas. Good growth with pointed leaf and yield potential of 4.5 t/ha. Fair establishment in seedbeds and field. Suitable for all areas and cures well to give predominantly orange grades. Resistant to wild fire (races 0and1), angular race, white mould, alternaria, tobacco mosaic virus and black shank.

Source: (TRB, 2012)

3.3 Seed storage environments

Tobacco seeds were harvested in August 2014 followed by cleaning, film coating and dressing with silver nitrate. The seed were equilibrated with ambient relative humidity of 33 % and

temperature of 20 °C which resulted in a seed moisture content of 7 % on dry-weight basis. Ten grams seed of each variety were then placed in hermetic dry storage at 5 ^{o}C and 20 °C to dry after-ripen. Every 2 weeks seeds were sampled from respective storage conditions and subjected to a germination test under specified imbibition solutions for each experiment.

3.4 Preparation of imbibition solutions

The phytohormones, 6-benzylaminopurine (BA) and gibberellic acid (GA) that were used in this study were purchased from Sigma Aldrich, Germany. One hundred milligrams of BA was dissolved in 5 ml of 1 molar sodium hydroxide (SkyLabs, South Africa) in a 1 L volumetric flask. This was then topped to the mark with distilled water to give a final concentration of 100 mg BA/L of water (Mukarati *et al.,* 2014). A 100 mg GA/L of water solution was prepared by first dissolving 100 mg of GA in 5 ml of 70 % ethanol (Hopkin and Williams, London) in a 1 L volumetric flask and then topping to the mark with distilled water (Mukarati *et al.,* 2014). A 5 mM hydrogen peroxide solution was prepared by adding 0.5 ml of 35 % hydrogen peroxide to a 1 L volumetric flask then topping to the 1 L mark with distilled water.

3.5 Germination tests and environments

Circular discs of cotton wool (Softex, Zimbabwe), 1 cm thick, were placed in 9 cm diameter disposable petri dishes (Carbi, South Africa). These were moistened with 16 ml of respective imbibition solution (Section 3.3) according to treatment specifications. Two layers of Whatman No. 1 filter paper (Whatman, England) with 100 by 25 mm^2 (5 mm x 5 mm) square grids were then placed on top of the moistened cotton. One seed was placed in each of the grids to give 100 seeds per petri dish. Seeded petri-dishes were then placed in 2 different germination chambers with

controlled temperatures of 20 ^{o}C and 30 ^{o}C. Two light regimes *viz* light and dark were established in each growth chamber, to give 4 environments (20 ^{o}C light and dark; 30 ^{o}C light and dark). To achieve dark conditions, petri dishes were covered with two layers of aluminum foil paper. Germination evaluations for dark conditions were done at ten days after experiment establishment (Mukarati *et al.,* 2014). Under light, seeds were exposed to 8/16 hour photoperiods, with light provided from T8 Diffuser Batten fluorescent lamps (Pierlite, Australia) for 8 hours and 16 hours of dar. Germination counts were done daily up to 10 days after experiment establishment for light incubated seeds and on the tenth day for seeds imbibed in dark. Experiments were run separately for each of the two varieties, following same basic procedures. Seed was considered to have germinated when the radicle was 1 mm long (Demir Kaya *et al.,* 2006). Germination parameters considered were total germination percent (TG) calculated as,

$$TG = \frac{total\ number\ of\ seeds\ germinated}{total\ number\ of\ seeds\ sown}\ x\ 100 \qquad (1)$$

and mean germination time (MGT) which was calculated to assess the rate of germination (R) using the formula of Ellis and Roberts (1981) recently used by Tavakkol *et al*. (2011).

$$MGT = \frac{\sum D\ x\ n}{\sum n} \qquad (2)$$

Where: n = number of seeds that germinated on the day (D), D = serial number of the day. Germination (R) which expresses the degree of germination over a designated time period as a percentage of the population based on the formula of Bewley and Black (1994) but recently used by (Mukarati *et al.,* 2014) was calculated as;

$$R = \frac{1}{MGT} \qquad (3)$$

CHAPTER 4

4.0 EFFECT OF AFTER-RIPENING TEMPERATURE AND IMBIBITION SOLUTIONS ON THE GERMINATION ON TOBACCO.

4.1 Introduction.

Seed dormancy is a mechanism by which seeds can inhibit their germination in order to wait for more favourable conditions (Finkelstein *et al.,* 2008). However, primary dormancy is caused by the effects of ABA that accumulates during seed development. Freshly harvested seeds either may not germinate completely, (Bewley, 1997) or are not able to germinate at temperatures higher than the optimum temperature. In tobacco germination occurs within the range of 18 to 23 ^{o}C (Hartely *et al.,* 2001) for seeds with dormancy, though ISTA (2003) pointed out that 20 - 30 ^{o}C can be used for germination evaluation. The timing of germination is important for the survival of seedlings since the seedling stage is the most vulnerable time in a plant's life cycle and germination should proceed even under stress environments such as increased temperatures and shading which can be conferred by the germination media such as pine bark and cocoa peat in the float tray system (Brandel and Schutz, 2005).

Rapid and uniform field emergence is a fundamental prerequisite for good crop establishment, especially under adverse environmental conditions (Subedi and Ma, 2005; Gupta *et al.,* 2008). The time from sowing to crop establishment is a crucial period in crop growth since it has a direct impact on final yield and quality (Gupta *et al.,* 2008). Techniques enhancing and stabilizing field emergence are the basis of crop success. Among them, seed after-ripening a pre-sowing dry storage method that reduces dormancy and widens the environmental window required for the germination. After-ripening improves germination through reducing the time from sowing to emergence thus improves seedling emergence uniformity. After-ripening influences the seed

germination response to light and temperature change during storage and this response is as a result of the changing level of seed dormancy (Brandel and Schutz, 2005). Temperature is regarded as a critical factor in regulating after-ripening and germination (Bouwmeester and Karssen, 1992; Bouwmeester and Karssen, 1993; Baskin and Baskin, 1986; Buhler *et al.*, 1997; Probert, 2000; Leon *et al.*, 2004). However the after-ripening process is slow and therefore several techniques have been proposed to complement the after-ripening process.

Endeavours to break the dormancy are centred on reducing the period of after-ripening required by the embryo (Zhou *et al.*, 2009). The first approach involves imbibition in H_2O_2. The other approach which reduces the period of after-ripening is by use of phytohormones such as gibberellic acid (GA) and benzyl adenine (BA). Since seed dormancy is incompletely understood and affects tobacco production, extensive experimentation is necessary to determine treatments that give near maximum germination.

4.2. Main objective

To evaluate effect of after-ripening temperature and imbibition solutions effect on tobacco seed germination performance under four different germination environments *viz* 20 ^{o}C light, 20 ^{o}C dark, 30 ^{o}C light and 30 ^{o}C dark

4.2.1 Specific objectives

1. To determine the effect of after-ripening temperature and imbibition solutions on germination rate of KRK 26 R and T71 tobacco seeds incubated for ten days in 20 ^{o}C light.

2. To determine the effect of after-ripening temperature and imbibition solutions on germination percentage of KRK 26 R and T71 tobacco seeds incubated for ten days in 30 ^{o}C light.

3. To determine the effect of after-ripening temperature and imbibition solutions on germination percentage of KRK 26 R and T71 tobacco seeds incubated for ten days in 20 ^{o}C dark.

4. To determine the effect of after-ripening temperature and imbibition solutions on germination percentage of KRK 26 R and T71 tobacco seeds incubated for ten days in 30 ^{o}C dark.

4.3 Hypothesis

1. After-ripening temperature and imbibition solutions affects germination rate of KRK 26 R and T71 tobacco seeds incubated for ten days in 20 ^{o}C light.

2. After-ripening temperature and imbibition solutions has effect on germination percentage of KRK 26 R and T71 tobacco seeds incubated for ten days in 30 ^{o}C light.

3. After-ripening temperature and imbibition solutions affect germination percentage of KRK 26 R and T71 tobacco seeds incubated for ten days in 20 ^{o}C dark.

4. After-ripening temperature and imbibition solutions has effect germination percentage of KRK 26 R and T71 tobacco seeds incubated for ten days in 30 ^{o}C dark.

4.4 Materials and methods.

4.4.1 Experimental design

Freshly harvested seeds of the two varieties, were placed in two storage environments (5 ^{o}C and 30 ^{o}C) and sampled every 2 weeks for 8 weeks to carry out germination tests carried out as outlined in Section 3.4. This experiment was a 2 x 4 factorial treatment experiment laid out in a Completely Randomized Design with four replications. After-ripening temperature had two levels (5 ^{o}C and 30 ^{o}C) and imbibition solutions had four levels (BA, GA, hydrogen peroxide and distilled water). With distilled water and 5 ^{o}C after-ripening as the control. The germination test was done in four germination environments, *viz* 20 ^{o}C light, 20 ^{o}C dark; 30 ^{o}C light and 30 ^{o}C dark and across the two varieties. Germination test of each variety under in each of the four of the environments was done separately and considered as a separate experiment.

4.4.2 Measurements.

Germination counts were taken daily up to ten days for light incubated seeds and was used to calculate the germination rate for 20 ^{o}C light incubated seed using the formulae illustrated in Section 3.4. For 30 ^{o}C light, 20 ^{o}C dark and 20^{o}C light incubated seeds, germination counts were taken on the 10th day and germination percentage was the parameter considered.

4.4.3 Statistical Analysis.

Data for germination percentage was transformed using Arc sin transformation where violation of Analysis of Variance (ANOVA) assumptions were attested. After normalizing the data,

ANOVA was done using the Genstat Stastical Package, Version 17. Treatment means were discriminated using Fischer' Protected Least Significant Difference test at 5 % level of probability.

4.5 Results

4.5.1: Effect of after-ripening temperature and imbibition solutions on germination rate of T71 tobacco seeds incubated for ten days in 20 ^{o}C light.

The was an interaction between after-ripening temperature and imbibition solutions on the germination rate of T71 from two weeks after storage throughout the eight week experimental period with ($P < 0.05$). The seeds imbibed in benzyl adenine had significantly lowest germination rate regardless of the after-ripening storage temperature at 2, 4, 6 and 8 WAS (Figure 4.1). At 2 WAS highest germination rate was attained in seeds imbibed in hydrogen peroxide and after-ripened at 30 ^{o}C. Seeds imbibed in GA also had significantly higher germination rate than the control (Fig 4.1). Seed after-ripened at 30 ^{o}C and germinated in water also had significantly higher germination rate than the control at 2 WAS.

At 4 WAS, seeds imbibed in hydrogen peroxide had considerably higher germination rate than the control. The combination of water and 30 ^{o}C after ripening also producing higher germination rate than the control. Seeds after-ripened at 30 ^{o}C and imbibed either in water or hydrogen peroxide germinated faster than the rest of other treatments and there were no significant differences between these two treatments (Fig 4.1). However seeds imbibed in any phyto-hormones showed lower germination rate than the control at 4 WAS.

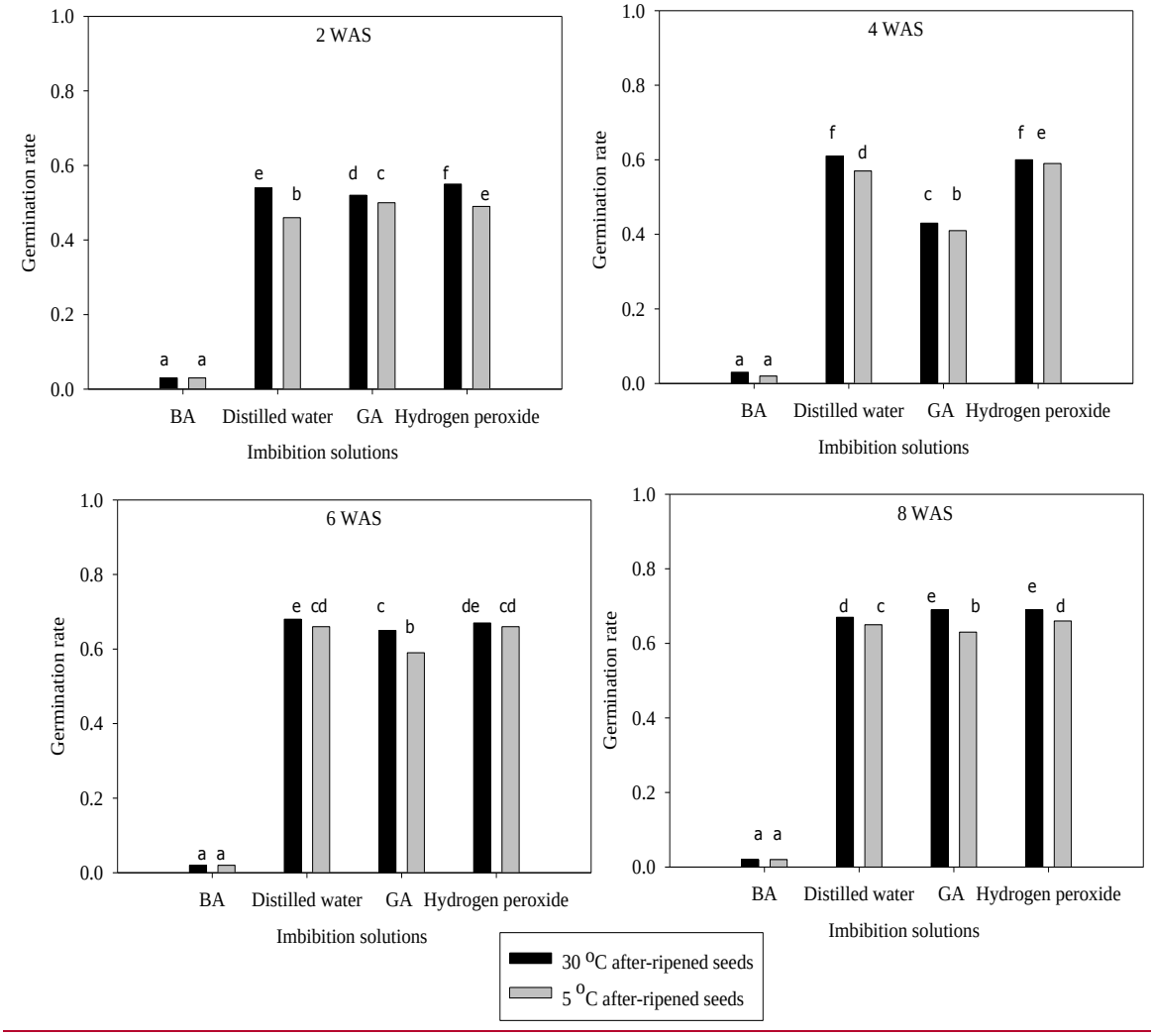

Figure 4.1: Effect of after-ripening temperature and imbibition solutions on germination rate of T71 seeds incubated for ten days in 20 ºC light after 2, 4, 6 and 8 weeks WAS. Bars with the same letter are not significantly different at 5 % level of significance.

The seeds after-ripened at 5 ºC and imbibed in GA showed lower germination rate than the control at 6 WAS (Figure 4.1). Unlike in all other storage durations, at 8 WAS the highest germination was attained in seeds after-ripened at 30 ºC and imbibed in gibberellic acid though this treatment combination wasn't different from seeds imbibed in hydrogen peroxide and after-ripened at the same temperature. GA+ 5 ºC after-ripening and BA imbibed seeds showed lower germination rate

than the control. There were no significant differences between water + 30 ^{o}C after-ripening treatment and hydrogen peroxide + 5 ^{o}C after-ripening and they both had significantly higher germination performance than the control at 8 WAS

4.5.2: Effect of after-ripening temperature and imbibition solutions on germination rate of KRK 26 R tobacco seeds incubated for ten days in 20 ^{o}C light.

To evaluate the effects of after-ripening temperature and imbibition solutions, seeds of KRK 26 R were incubated at 20 ^{o}C in the presence of light. There was interaction effect of after-ripening temperature and imbibition solutions throughout the experimental period i.e. 2-8 WAS ($P< 0.05$) on germination rate of KRK 26 R seeds. The 30 ^{o}C after-ripening temperature significantly improved germination rate as compared to 5 ^{o}C after-ripened seeds regardless of the imbibition solution except in BA imbibed seeds at 2, 4 and 6 WAS imbibed seeds where which showed no significant differences (Fig 4.2). At 2, 4 and 6 WAS seeds dry stored at 30 ^{o}C and imbibed in either hydrogen peroxide or water had the highest germination as measured by the germination rate and there were no significant difference between the two treatments (Fig 4.2).

At 8 WAS the highest germination rate was attained in seeds after-ripened at 30 ^{o}C + followed by water imbibition. At 6 and 8 WAS, 5 ^{o}C after-ripening + hydrogen peroxide had significantly lower than the control with the opposite of this happening at 2 and 4 WAS. However, the phyto-hormones imbibed seeds continuously showed reduced germination performance throughout all the WAS

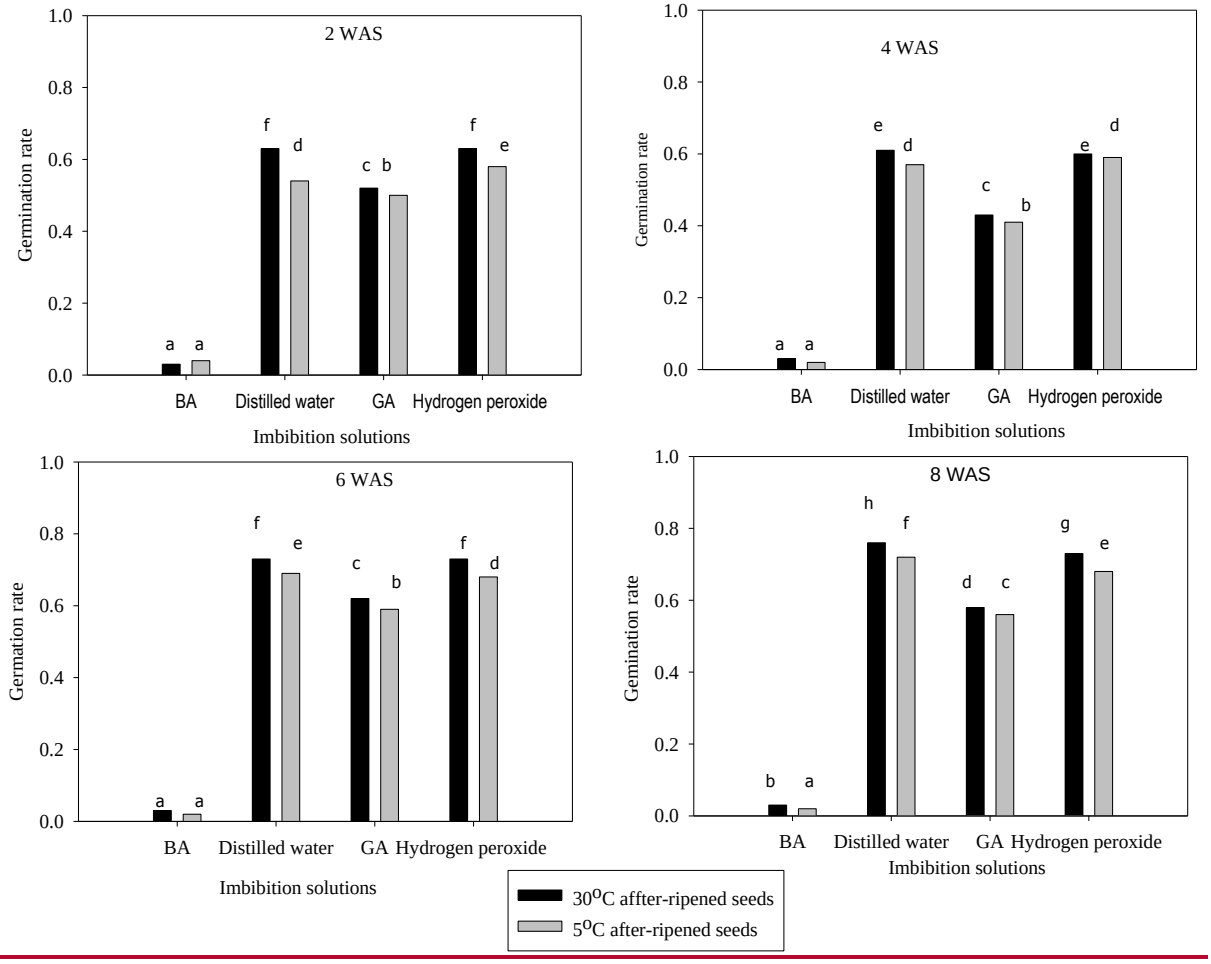

Figure 4.2: Effect of after-ripening temperature and imbibition solutions on germination rate of KRK 26 R seeds incubated for ten days at 20 ºC in the presence of light after 2, 4, 6 and 8 WAS. Bars with the same letter are not significantly different at 5 % level of significance

4.5.3 Effect of after-ripening temperature and imbibition solutions on germination percentage of T71 tobacco seeds incubated for ten days in 30 ºC light.

There was an interaction between after-ripening temperature and imbibition solutions on germination percentage T71 tobacco seeds at 2, 4, 6 and 8 WAS ($P < 0.05$). Seeds imbibed in gibberellic acid had significantly higher germination percentages than seed imbibed in any other

solutions. Seeds after-ripened at 30 ^{o}C and imbibed in gibberellic acid constantly germinated better than the other treatment combinations throughout all the WAS (Fig 4.3). The best germination of 83.07 %, 81.55 %, 80.70 % and 81.78 % for week 2, 4, 6 and 8 WAS respectively (Fig. 4.3) was attained for the treatment GA + 30 ^{o}C after-ripening.

Like in all other experiments BA inhibited seed germination. At 2 WAS there were no significant differences between the control and hydrogen peroxide imbibed seeds after a 5 ^{o}C after-ripening seeds on germination percentage. The 30 ^{o}C after-ripened seed + water and GA imbibed seeds had significantly higher germination percentage than the control. BA imbibed seeds after-ripened 5 ^{o}C after-ripening and 5 ^{o}C after-ripening + hydrogen peroxide had significantly lower germination percentage than the control. However all other treatments had better germination percentage than the control at 4 and 8 WAS. At 6 WAS, seeds imbibed in hydrogen peroxide and BA showed lower germination percentage than the control. It is also noted that, except for a few cases where germination percentage is similar for 30 ^{o}C and 5 ^{o}C (i.e. for hydrogen peroxide at 2 WAS and 6 WAS; GA and BA at 8 WAS), germination percentage for all solutions were significantly higher in 30 ^{o}C after-ripened seeds than in 5 ^{o}C after-ripened seeds.

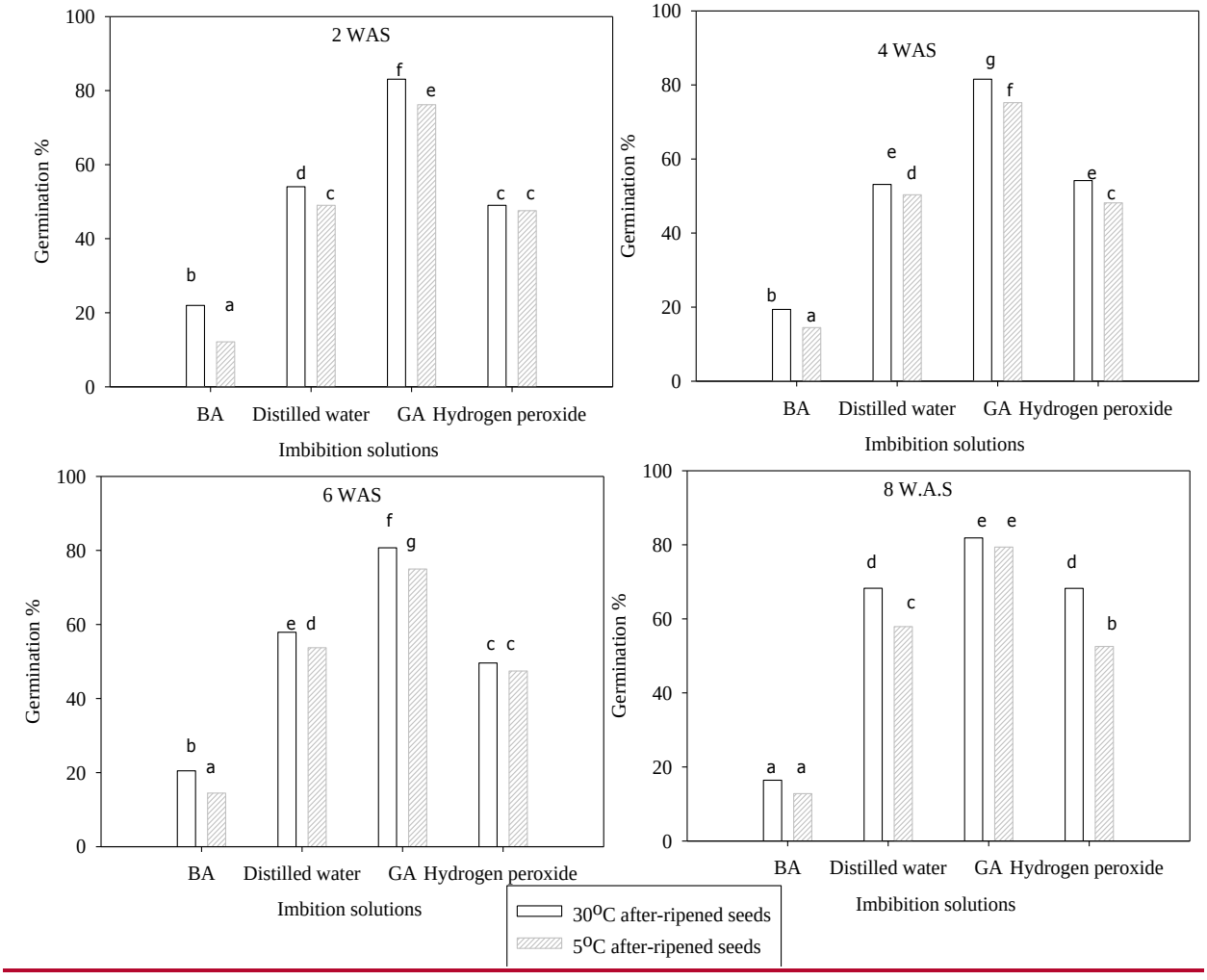

Figure 4.3: Effect of after-ripening temperature and imbibition solutions on germination percentage of T71 seeds incubated for ten days in 30 ºC light after 2, 4, 6 and 8 WAS. Bars with the same letter are not significantly different at 5 % level of significance.

4.5.4 Effect of after-ripening temperature and imbibition solutions on germination percentage of KRK26 R tobacco seeds incubated for ten days in 30 ºC light.

After-ripening temperature and imbibition solutions had an interaction effect on KRK 26 R seeds germination percentage in all the four sampling WAS ($P < 0.05$). The 30 ºC after-ripening and

gibberellic acid combination maintained the highest germination percentages at all stages except at 8 WAS this treatment had no significant difference with GA imbibed + 5 ^{o}C after-ripened seeds.

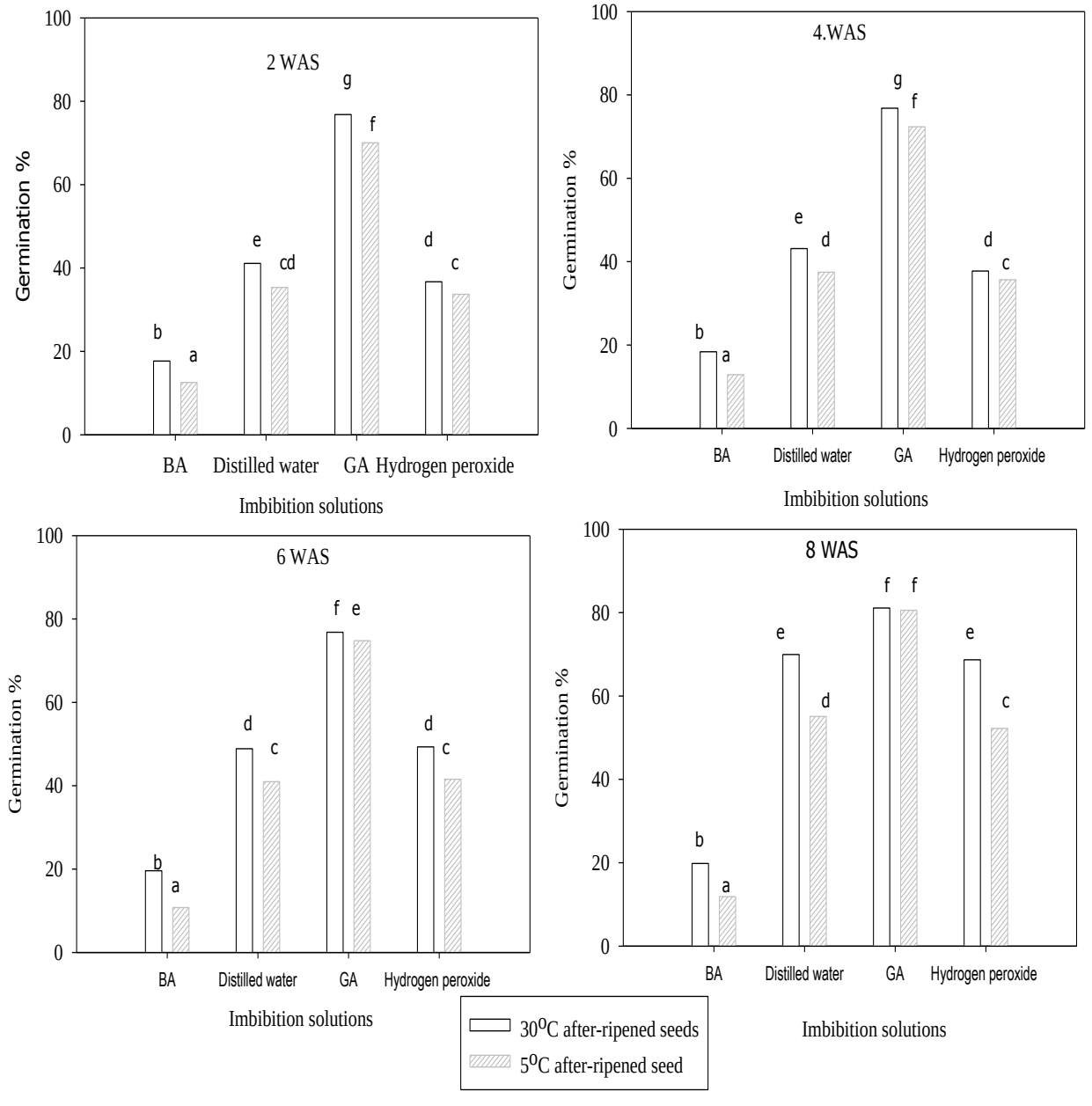

Figure 4.4: Effect of after-ripening temperature and imbibition solutions on germination rate equivalent of KRK 26 R seeds incubated for ten days in 30 ^{o}C light after 2, 4, 6 and 8 WAS. Bars with the same letter are not significantly different at 5 % level of significance.

Seed after-ripened at 30 ^{o}C and imbibed in distilled water had significantly higher germination percentage than the control at 2, 4, 6 and 8 WAS. BA resulted in significantly lower germination percentage than the control throughout these conditions (Fig. 4.4). There were no significant differences between the control and hydrogen peroxide imbibed seeds at 2 WAS and hydrogen peroxide + 30 ^{o}C dry stored seeds at 4 WAS. However there were significant lower germination percentage noted in seeds imbibed in hydrogen peroxide and after-ripened at 5 ^{o}C at 4 and 8 WAS differences as compared to the control.

At 2 and 6 WAS there were no significant differences between the control and seeds imbibed in hydrogen + after-ripened at 5 ^{o}C. However the hydrogen peroxide + a 30 ^{o}C after-ripening treatment had significantly higher germination percentage than the control at 6 and 8 WAS (Fig. 4.4). Seed after-ripened at 30 ^{o}C and imbibed in water had no significant difference with hydrogen peroxide imbibed at 6 WAS. Similar to earlier observation for T71, germination percentage s significantly higher at 30 ^{o}C after-ripened seed than at 5 ^{o}C after-ripened seed for all solutions at all stages (WAS) except for GA at 8 WAS when germination percentage similar for the two after-ripening temperature regimes.

4.5.5 Effect of after-ripening temperature and imbibition solutions on mean germination percentage of T71 tobacco seeds incubated for ten days in 20 ^{o}C dark.

There was a significant interaction between the after-ripening temperature and imbibition solution at 2 and 4 ($P < 0.005$). However, there was no interaction at 6 and 8 WAS ($P > 0.05$). Seeds after-ripened at 5 ^{o}C and imbibed in either benzyl adenine or hydrogen peroxide did not differ significantly from the control (Fig. 4.5), on germination percent at 2 WAS. Benzyl adenine + 30

^{o}C dry stored seeds also had no significant differences with the control .Seeds after-ripened at 30 ^{o}C and imbibed in hydrogen peroxide had no differences with the control (Fig. 4.5) at 2 WAS. After-ripening seeds at 30 ^{o}C and imbibing in either water or hydrogen peroxide outperformed the control. GA resulted in higher germination performance regardless of the after-ripening temperature with the highest germination attained from GA + 30 ^{o}C after-ripened seeds at 2 WAS. At 4 WAS BA+ 5 ^{o}C after-ripening showed no significant differences with the control. However seeds imbibed in hydrogen peroxide and GA imbibed seeds had significantly higher germination percentage than the control. A 30 ^{o}C after-ripening + BA or and 30 ^{o}C after-ripening + water gave significantly higher germination performances than the control at 4 WAS.

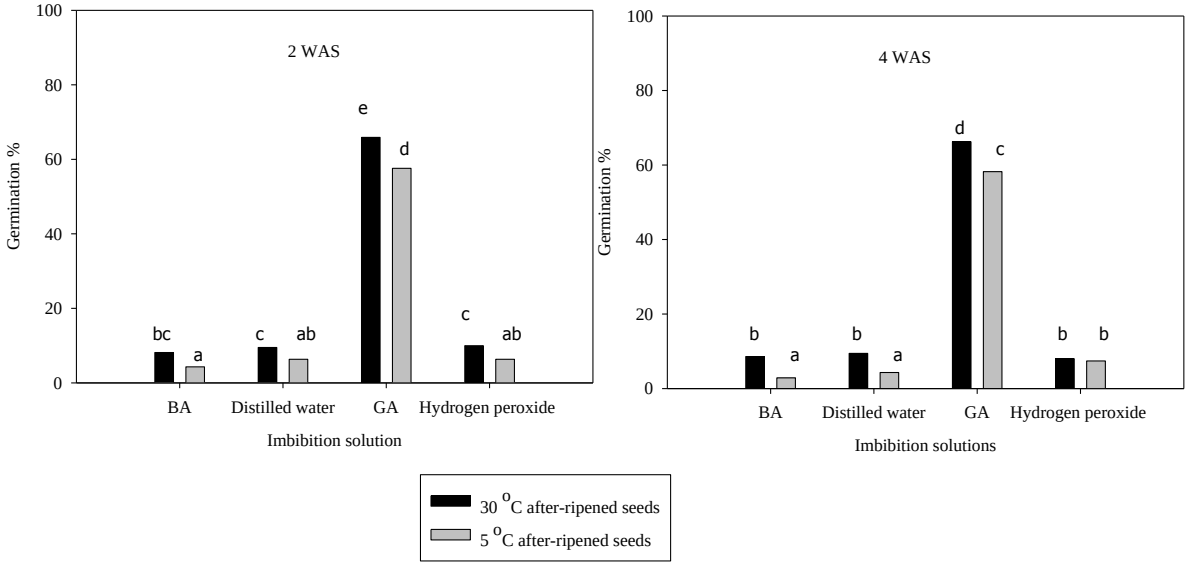

Figure 4.5: Effect of after-ripening temperature and imbibition solutions on germination percentage of T71 seeds incubated for ten days in 20 oC dark after 2 and 4 WAS. Bars with the same letter are not significantly different at 5 % level of

There was no significant interaction between after-ripening temperature and imbibition solution on germination at 6 and 8 WAS, however there was significant effect of after-ripening

temperature main effect ($P < 0.05$) and imbibition solution ($P < 0.05$) main effect at 6 and 8 WAS. Seeds after-ripened at 30 ^{o}C had significantly higher germination rate than the seeds after-ripened at 5 ^{o}C (Table 4.1). At 6 and 8 WAS there were significant differences among the imbibition solution with BA showing significantly lower germination percentage than the rest of the imbibition solutions (Table 4.1). However, there was no significant difference between the water and hydrogen peroxide on germination percentage of T71 seeds (Table 4.1). Gibberellic acid imbibed seeds had significantly higher germination percentages than the rest of the imbibition solutions.

Table 4.1: Effect of after-ripening temperature and imbibition solutions on germination percentage of T71 seeds incubated for ten days in 20 ºC dark after 6 and 8 WAS.

	Germination %	
Factor	6 W.A.S	8 W.A.S
After-ripening temperature		
5 ºC	25.48[a]	27.89[a]
30 ºC	30.03[b]	32.53[b]
P value	<0.001	<0.001
SED	0.494	0.415
Imbibition solutions		
Benzyl adenine	9.53[a]	9.80[a]
Distilled water	19.83[b]	21.56[b]
Gibberellic acid	61.20[b]	67.36[c]
Hydrogen peroxide	20.46[b]	22.11[b]
P value	<0.001	<0.001
S.E.D	0.699	0.587
CV %	5.0	3.9

Values followed by the same superscripts not significantly different at 5 % level of significance

4.5.6 Effect of after-ripening temperature and imbibition solutions on mean germination percentage of KRK 26 R tobacco seeds incubated for ten days in 20 ºC dark.

There was no interaction between imbibition solutions and after-ripening temperature at 2 and 4 WAS ($P > 0.05$) on germination percentage of KRK 26 R seeds. However the main effects *viz*

imbibition solutions and after-ripening temperature showed significant differences ($P < 0.05$) at the two sampling periods (Table 4.2). Similar to T71 gibberellic acid imbibed seeds had the highest germination percentage at 2 WAS (62.07%) and 4 WAS (65.32%).The highest germination performance was attained in seeds after-ripened at 30 ^{o}C than in seed after-ripened at 5 ^{o}C both at 2 and 4 WAS (Table 4.2).

AT 6 and 8 WAS, after-ripening x imbibition solutions interaction was noted ($P < 0.05$). Seeds subjected to GA+ 30 ^{o}C after-ripening had significantly the highest germination percentages of 73.88% and 74.15% at 6 and 8 WAS respectively (Fig. 4.6). Benzyl adenine + 5 ^{o}C after-ripened seeds constantly showed lowest germination percentages regardless of the after-ripening temperature. No significant differences were recorded at 6 WAS between the control and hydrogen peroxide imbibed + 5 ^{o}C after-ripening combination (Fig 4.6). After ripening seed at 30 ^{o}C followed by imbibition in either water or hydrogen peroxide significantly stimulated better germination than the control with the highest germination being attained in GA + 30 ^{o}C after-ripened seed (Fig 4.6).

Table 4.2: Effect of after-ripening temperature and imbibition solutions on germination percentage of KRK 26 seeds incubated for ten days in 20 ºC dark at 2 and 4 WAS.

	Germination %	
Factor	2 W.A.S	4 W.A.S
After-ripening temperature		
5 ºC	18.00[a]	19.55[b]
30 ºC	24.05[b]	23.20[a]
P value	<0.001	<0.001
S.E.D	0.665	0.653
Imbibition solutions		
Benzly adenine	5.66[a]	5.73[a]
Distilled water	7.63[b]	6.45[ab]
Gibberellic acid	62.07[b]	65.32[c]
Hydrogen peroxide	8.74[b]	7.99[b]
P value	<0.001	<0.001
S.E.D	0.940	0.923
CV %	8.9	8.6

Values followed by the same superscripts not significantly different at 5 % level of significance.

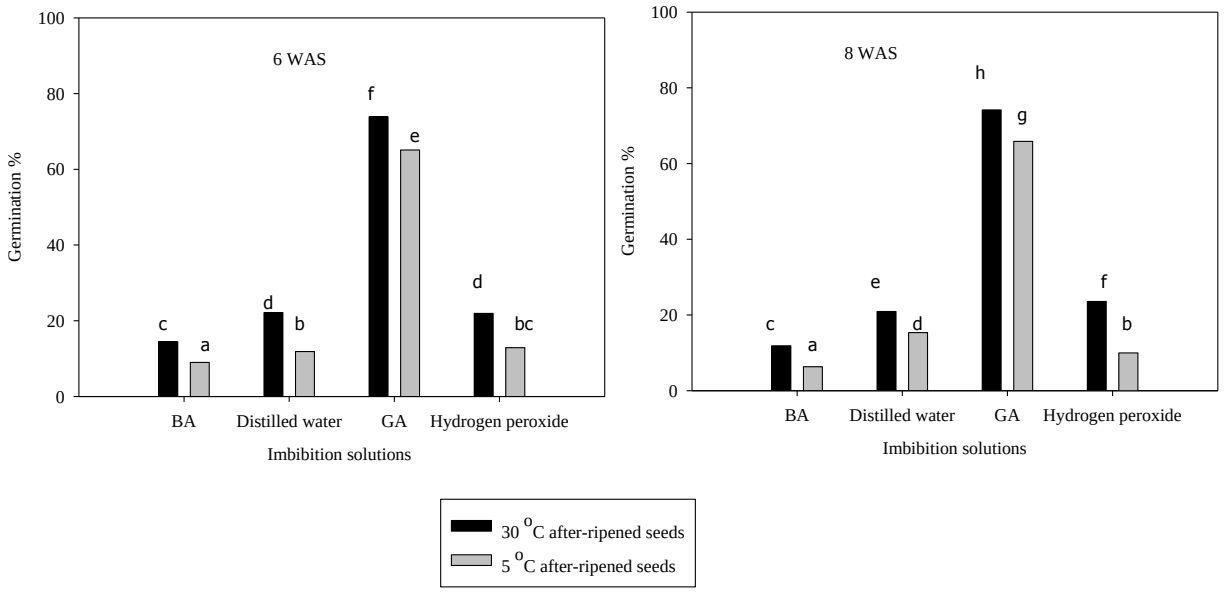

Figure 4.6: Effect of after-ripening temperature and imbibition solutions on germination percentage of T71 seeds incubated for ten days in 30 ºC dark at 6 and 8 WAS. Bars with the same letter are not significantly different at 5 % level of significance.

4.5.7 Effect of after-ripening temperature and imbibition solutions on mean germination percentage of T71 tobacco seeds incubated for ten days in 30 ºC dark.

After ripening temperature and imbibition solution interacted on germination percentage of T71 dark incubated seeds in all the four sampling points ($P < 0.05$). Throughout all WAS, BA significantly reduced germination of T71 seeds while GA gave the highest germination percent regardless of the after-ripening temperature with the highest germination percentage of 51.0%, 62.25%, 65.67% and 68.08% at 2, 4, 6 and 8 WAS respectively (Fig. 4.7). Hydrogen peroxide seeds showed no significant differences with the control at 2 WAS. Water and + 30 ºC after-ripening significantly increased germination percentage than the control at 4 WAS.

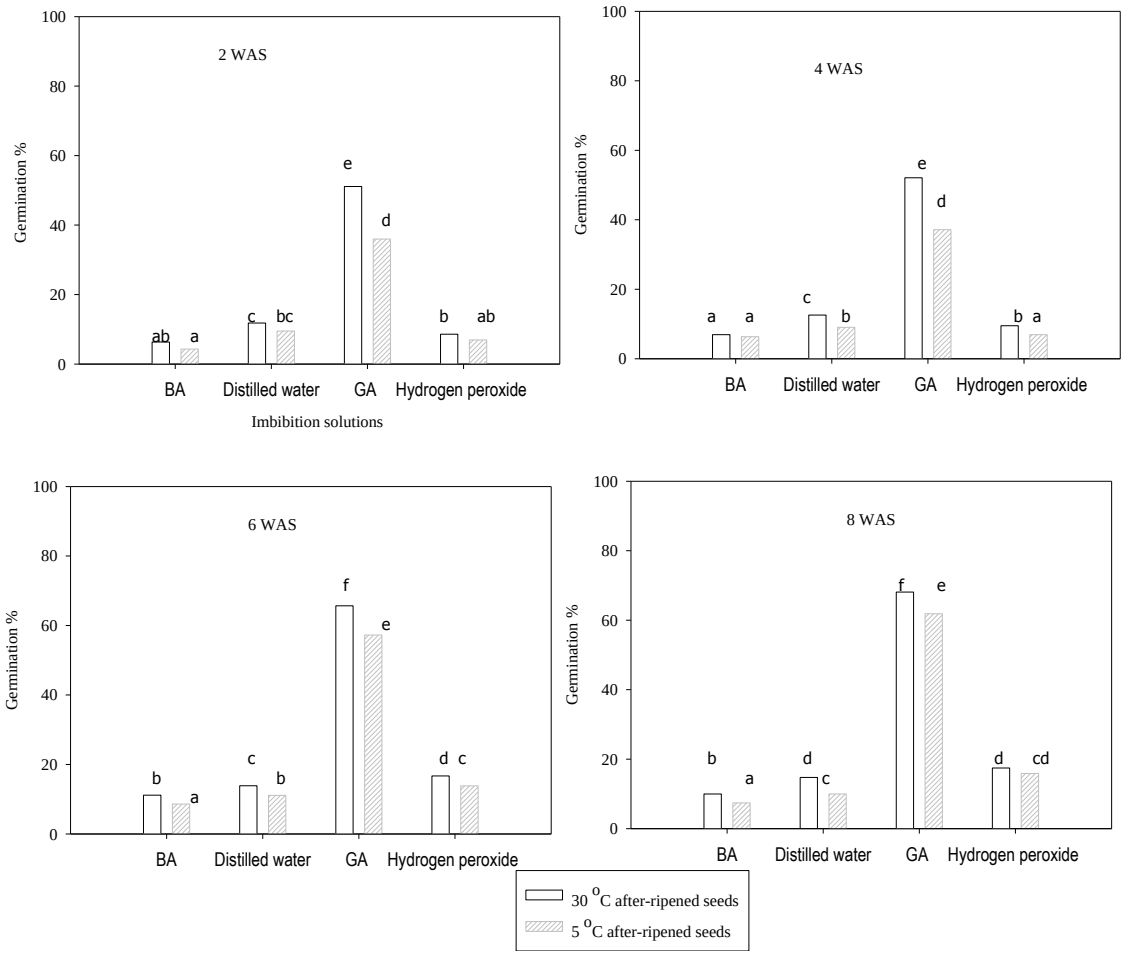

Figure 4.7: Effect of after-ripening temperature and imbibition solutions on germination percentage of T71 seeds incubated for ten days in 30 ºC dark at 2, 4, 6 and 8 WAS. Bars with the same letter are not significantly different at 5 % level of significance.

There were significantly lower germination percentage in seeds treated with BA + 5ºC after-ripening. Seeds imbibed either in hydrogen peroxide or water after after-ripened at 30 ºC slightly improved germination percentage at 6 WAS. BA imbibed seeds showed lower germination percentages than the control at 8 WAS. The 5 ºC after-ripening + hydrogen peroxide imbibition had comparable results with the control at 8 WAS. Seeds after-ripened at 30 ºC and imbibed either in water or hydrogen peroxide showed marginal increase in germination percentages at 8 WAS.

4.5.8 Effect of after-ripening temperature and imbibition solution on mean germination percentage of KRK 26 R tobacco seeds incubated for ten days in 30 ^{o}C dark.

After-ripening temperature and imbibition solution had an interaction on germination of KRK 26 R seeds incubated at 30 ^{o}C under dark conditions ($P < 0.05$) at 2, 4, 6 and 8 WAS. Like with T71 seeds, GA imbibed seeds consistently improved germination with highest germination percentage attained in GA + 30 ^{o}C dry stored seeds. There were comparable results between the control, seeds imbibed in BA and seeds imbibed in hydrogen peroxide + a 5 ^{o}C after-ripening at 2 WAS (Fig 4.8). The 30 ^{o}C after-ripened seeds imbibed in either water or hydrogen peroxide marginally improved germination percentage of KRK 26 R seeds at 2 WAS. At 4 WAS no significant differences were recorded between BA + 5 ^{o}C after-ripening and the control. Seeds after-ripened at 30 ^{o}C and imbibed in either water or BA showed slight increase on germination percentages at 4 WAS.

There were comparable results between seed treated with BA + 5 ^{o}C after-ripening, hydrogen peroxide + 5 ^{o}C after-ripening and the control on germination percentage at 6 WAS. The 30 ^{o}C after-ripened seeds imbibed in either hydrogen peroxide or BA at 6 WAS significantly improving germination in KRK 26 R. Nevertheless at 8 WAS there was no significant difference between 5 ^{o}C after-ripened seeds imbibed in either benzyl adenine or hydrogen peroxide on germination performance. 30 ^{o}C after-ripened seeds and imbibed either in water, BA or hydrogen peroxide increased germination percentages slightly at 8 WAS (Fig. 4.8). Comparing the two after-ripening temperature regimes there is a notable increase in germination percentage in seeds after-ripened at 30 ^{o}C the 5^{o}C after-ripened seed expect in BA imbibed seed at 2 WAS and hydrogen peroxide at 4 WAS where the comparable results were attained

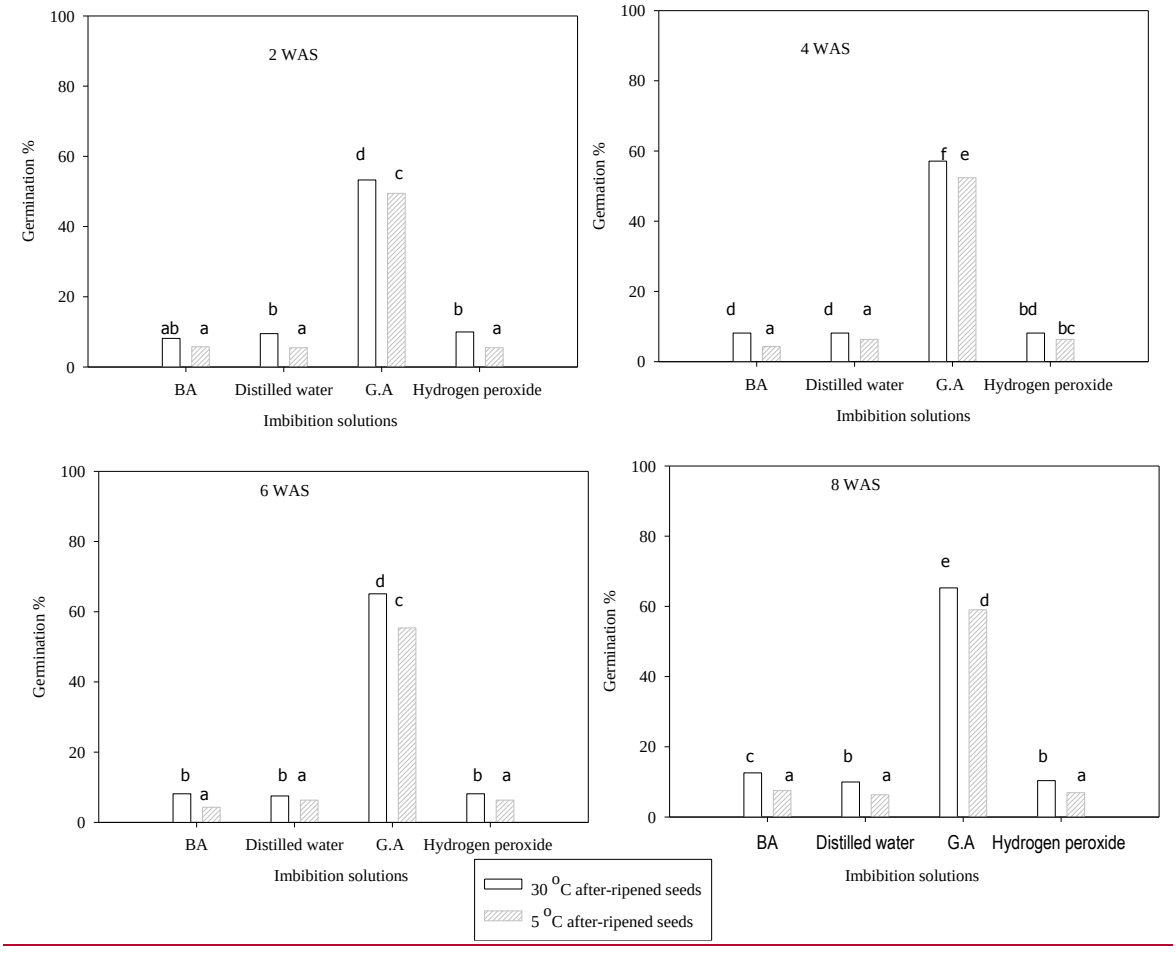

Figure 4.8. Effect of after-ripening temperature and imbibition solutions on germination percentage of KRK 26 seeds incubated for ten days in 30 ºC dark after 2, 4, 6 and 8 weeks after storage (WAS). Bars with the same letter are not significantly different at 5 % level of significance.

4.6 Discussion.

Since transplants are used to establish the tobacco crop, it is extremely important that uniform seed germination occurs. In light of this germination of freshly harvested seeds of T71 and KRK 26 R seeds was determined to assess its relationship with germination rate and germination percentage under both light and dark conditions at two constant temperature levels of 20 ^{o}C and 30 ^{o}C. Freshly harvested seeds of two tobacco cultivars were incubated for ten days.

4.6.1. Effect of after-ripening temperature on tobacco seeds germination

Seeds act as environmental sensors and adjust their dormancy status as a response to a range of temperatures (Finch-Savage and Leubner-Metzger 2006; Graeber *et al*, 2012). After-ripening results in a widening of the environmental conditions that allow germination and consequently increases the germination speed and rate (Finch-Savage and Leubner-Metzger, 2006). Temperature alters the rate of dormancy alleviation during dry storage and it's a critical environmental factor that affects the status of dormancy during dry storage (Probert, 2000). In this study it is evident that dormancy is alleviated and germination stimulated faster in 30 ^{o}C after-ripened seeds as compared to seeds after ripened at 5 ^{o}C in most experiments. This positive influence of increasing after-ripening temperature and dormancy release is in agreement with the metabolic theory of ecology postulated by Brown *et al.* (2004), which allude to the fact that the temperature dependence of most biological reaction rates follows and obeys the Arrhenius relationship (Gillooly *et al.*, 2001).

These results are in agreement with Bazin *et al*. (2011) who reported an improved germination rate of sunflower seeds after-ripened at 20-25 ^{o}C as compared to seeds after-ripened at 5, 10 and 15 ^{o}C. Shutz *et al.* (2002) reported an improved germination percentage with the increase in after-ripening

temperature from 5, 10, 15, 20, 25 ^{o}C in seeds of four *Asteraceae*. Murdoch and Ellis (2000) concluded that after-ripening is promoted (excluding dry heat treatment) by temperatures in the range of 25-75 ^{o}C. Peish *et al,* (1998) also found out that increasing the after-ripening temperature to 35 ^{o}C increased germination performance as compared to 25, 20, 15 and 10 ^{o}C in three *Asteraceae.* During after-ripening of tobacco seeds there is evidence of enzymatic and no enzymatic reaction and their rate is dependent on temperature. Increased temperature such as 30 ^{o}C during after-ripening increase of production of β-1,3-glucanase, mRNA abundance and subsequent increase in Glu enzyme activity (Leubner-Metzger, 2003; 2005).

The enzyme β-1,3-glucanase is responsible for endosperm weakening a perquisite for germination in tobacco. Therefore this might be the reason why there was an increased germination performance of seeds after-ripened at 30 ^{o}C as compared to 5 ^{o}C after-ripened seed. Reactive oxygen species (ROS) production are also enhanced by increased temperatures and have profound effect on endosperm weakening thus enhances seed germination. Increased temperatures also are responsible for membrane alterations thus facilitate radicle protrusion. Both the ROS production and membrane weakening are facilitated by increased temperature, this might be the reason why there were increased germination performance of seeds after-ripened at 30 ^{o}C as compared to 5 ^{o}C after-ripened seed. (Bailly, 2004; Borghetti *et al.,* 2002; Hallett and Bewley, 2002).

4.6.2 Effect of after-ripening temperature and imbibition solution on germination rate of T71 and KRK 26 R tobacco seeds incubated for ten days in 20 ^{o}C light.

Seeds after-ripened at 30 ^{o}C and imbibed either in hydrogen peroxide or water had noticeable improvements in germination rate for the two varieties. The effect of the increased germination performance in seeds imbibed in water after 30 ^{o}C after-ripening and can be attributed to the effect

of after-ripening temperature as described in section 4.4.1 since the water imbibed seeds after ripened at lower temperature (5 ^{o}C) had reduced germination performance under 20 ^{o}C light conditions. However in this contemporary study, it was observed that there was significantly higher germination rate in seeds imbibed in hydrogen peroxide and after-ripened at 30 ^{o}C as compared to the control.

In many crop plants hydrogen peroxide is regarded as a toxic molecule due to its highly oxidative reactivity especially in presence of catalytic metal ions such as iron and copper. It produces hydroxyl radical which oxidizes cell components (Ogawa and Iwabuchi, 2001). However, exogenously applied hydrogen peroxide ameliorates seed germination in many plants (Fontaine *et al.,* 1994; Korytov and Nirimanov 1997; Wang *et al.,* 1998; Cavusoglu and Kabar, 2010). The improved germination rate of hydrogen peroxide can be elucidated by the fact that the scavenging activity for H_2O_2 which results in the production of oxygen needed for mitochondrial respiration through the activation of the oxidative pentose phosphate pathway. Another explanation is that hydrogen peroxide cracks the hard seeds coats thus allowing them to interact with water.

Hydrogen peroxide also inactivates ABI1 and ABI2 type 2C protein phosphatase, enzymes that function in ABA signalling (Meinhard *et al.,* 2002) thus reducing the inhibitory effect of ABA. Hydrogen peroxide also influences seed germination through the reaction with either oxygen or oxygen radicle in the presence of cell wall peroxidases, leading to the formation hydroxyl ions (Chen and Schopfer, 1999). The hydroxyl ions then act on the cell wall by causing polysaccharide cleavage resulting in endosperm weakening through degradation of cell wall polysaccharides such as pectin and xyloglucan which provides restriction to radicle protrusion. Liu *et al.* (2010) similarly found that after seven days of imbibition in 5 mM hydrogen peroxide, germination percentage was promoted in freshly harvested Arabidopsis seeds with much higher concentration such as 100 mM

showing less effect. In sunflower (*Helianthus annuus*) hydrogen peroxide also improved germination and seedling growth (Waheed et al., 2008).

Normally the application of phyto-hormones such as GA and BA is explored in promoting germination in poorly germinating seeds (Bewley, 1997; Yamaguchi and Kamiva, 2001; Jacobsen *et al.,* 2002; Joshi *et al.,* 2010). Surprisingly GA showed lower germination rate than the control expect at 2 and 8 WAS in T71 with BA constantly inhibiting germination throughout this study. This increased germination in GA imbibed seeds under 30 ^{o}C light at 8 WAS can be attributed to increased sensitivity to exogenous GA due to the lengthening of after-ripening duration as suggested by Finch-Savage and Leubner-Metzger (2006).

Explanation on the lack of the stimulatory effect of BA and GA on tobacco seeds is not clear, however these results are not unique. Wall *et al.* (2010) also reported that gibberellic acid failed to improve germination in *Pyxidanthera brevifolia.* These results are also consistent with the findings of Yang *et al.* (2013) in *Galax urceolata* (Brummitt) where gibberellic acid reduced germination percentage from 49 % to around 30 %. da Silva *et al.* (2005) reported that gibberellic acid can actually be detrimental and they concluded that gibberellic acid inhibits coffee (*Coffea arbica* cv Rubi) seed germination and consequently causing cell death in the embryo.

Contrary to many reports on the stimulatory effect of BA, it also inhibits radicle protrusion as reported by Valio (1976), Takaki and Dietrich, (1979) and Gottfried and Heidmann (1992). From these results and others' it is possible that tobacco seeds have enough endogenous GA and BA to initiate, sustain and complete germination under 20 ^{o}C light conditions. So additional exogenous BA and GA (expect for 2 and 8 WAS in T71) is superfluous, though this warrants further investigation.

4.6.3 Effect of after-ripening temperature and imbibition solutions on germination percentage of T71 and KRK 26 R tobacco seeds incubated for ten days in 30 ºC light.

In tobacco seed germination the optimum germination falls within the range 18 to 23 ºC, however, .for seeds in quiescence germination should occur up to 30 ºC (Hartely *et al.,* 2002). Seed with some level of dormancy shows reduced germination performance under this temperature due to low levels of GA, transient increase in abscisic acid (ABA) and reduction in ethylene production (Gonai *et al.,* 2004). In tobacco seed increased ABA content enhances seed dormancy or delays germination. In seeds incubated at 30 ºC, there is conspicuous promotion of germination in GA imbibed seeds. This is might be due to the fact that exogenous GA counteracts the ravaging effect of increased ABA by reducing ABA metabolism biosynthesis and enhancing ABA catabolism thus enhancing germination (White, 2000). This might be the explanation why there is marked reduction in germination of seeds except for GA imbibed seeds. At 30 ºC

Similar results have been reported where exogenous GA was found to antagonistically affect ABA mediated germination under increased temperature in beechnut (Lorenzo *et al.,* 2001) and maize (White *et al.,* 2000). In lettuce seeds ABA concentration increased 100 fold at 30 ºC as compared to 15 ºC and the application of exogenous GA promoted germination through reduction in endogenous ABA content at 30 ºC. Unlike under 20 ºC incubated seeds imbibed in hydrogen peroxide did not give satisfactory results. Wahid *et al.* (2007), reported that increased temperatures stimulated the production of reactive oxygen species, thus addition of hydrogen peroxide provides net increase in the hydrogen peroxide concentration which can be detrimental to seed germination. It is possible that this increase is the reason for reduced germination in hydrogen peroxide imbibed seeds.

4.6.4 Effect of after-ripening temperature and imbibition solutions on germination percentage of T71 and KRK 26 R tobacco seeds incubated for ten days in 20 ºC and 30 ºC dark.

Under dark conditions GA constantly improved germination regardless of the after-ripening temperature. In tobacco seeds, germination in dark is impassable at a step before testa rupture and limited testa nor endosperm rupture occur even after several weeks of dark imbibition (Leubner-Metzger *et al.,* 1996, Leubner-Metzger, 2002). Red light activates the phytochrome signal transduction pathway, resulting in the release of dormancy and the promotion of germination (Emmler and Schafer, 1997; Leubner-Metzger, 2002). GA can substitute for the red-light trigger required for dormancy release and subsequent germination thus it can promote germination of tobacco seeds incubated in the dark (Leubner-Metzger *et al.,* 1996, Leubner-Metzger, 2002 Peng and Harberd, 2002). This can be the reason why there is increased germination of dark imbibed seeds.Similar results have also been reported in lettuce where exogenous application of GA enhanced germination percentage under 30 ºC light (Yamaguchi *et al.,* 2001).

Leubner-Metzger (2002) reported that apart from substituting the light requirement GA also is involved in inducing dark germination of dormant tobacco seeds through the promotion of ABA-delayed endosperm rupture in dark-imbibed quiescent tobacco seeds and osmoticum-delayed testa and endosperm rupture of light-imbibed seeds (Leubner- Metzger *et al.,* 1996). This ABA delay was successfully reversed by exogenous GA in *Nicotiana plumbaginifolia* through stimulation of ABA degradation and inhibition of ABA synthesis (Grappin *et al.,* 2000).

The sensitivity of exogenous applied GA increased with the increase in after-ripening duration in this study. These results are in agreement with the findings of Leubner-Metzger (2002), who reported that the GA sensitivity differs with the increase in after-ripening time.

4.7 Conclusion and Recommendations.

From this study the following conclusions and recommendations can be made about i) tobacco seeds germination rate under light + 20 ^{o}C ii) germination percentage under dark conditions and iii) germination percentage under light conditions.

i) Germination rate for seed incubated at 20 ^{o}C light.

After-ripening tobacco seeds at 30 ^{o}C and imbibing seeds in either hydrogen peroxide or water is more effective in promoting germination rate under 20 ^{o}C light and after-ripening seeds at 30 ^{o}C improves germination than 5 ^{o}C. However there is no merit in addition of phyto-hormones to promote germination under this conditions except in T71 seeds at 2 and 8 WAS. There is more molecular work needed to assess the unsatisfactory effect of seed germination rate in phyto-hormone imbibed seeds.

ii) Germination percent of dark incubated tobacco seeds

Gibberellic acid enhances germination of the two varieties in dark at 20 ^{o}C and 30 ^{o}C with after-ripening seeds at 30 ^{o}C slightly improving germination. In can be concluded that germination of tobacco is light dependent. Therefore farmers are recommended to after-ripening temperature their seeds at 30 ^{o}C and bury seeds at correct depths to allow maximum exposure to light.

iii) Germination of 30 ^{o}C light incubated tobacco seeds.

Tobacco seeds germination percent is reduced under 30 ^{o}C though germination percentage and germination can be enhanced by gibberellic acid imbibition or after-ripening at 30 ^{o}C. The increase in the after-ripening period provides improved germination especially for water imbibed seeds. It is recommended that tobacco seeds be after-ripened at 30 ^{o}C if gibberellic acid is not used.

CHAPTER 5

5.0 EFFECT OF AFTER-RIPENING TEMPERATURE AND IMBIBITION SOLUTIONS ON β -1, 3-GLUCANASE ACTIVITY OF KRK 26 R AND T71 TOBACCO SEEDS.

5.1 Introduction.

The adoption of improved tobacco varieties is being hindered by poor seed germination (Kuboja *et al.,* 2011) leading to reduced tobacco productivity. Seed germination sometimes fail to be steadfast due to combined effect of seed dormancy and environmental conditions (Gupta *et al.,* 2008; Tavakkol *et al,* 2011). Seed dormancy is of both agricultural and ecological importance since it lengthens seed longevity (Wang *et al.,* 2007; Tavakkol *et al,* 201). In a desolate environment, seed dormancy has profound effects on the survival and establishment of different innate plant species (Tavakkol *et al,* 2011). Dormancy has profound effects on homogeneity of germination of field crops (Wang *et al.,* 2007), thus affect tobacco seedling production consequently it has to be completely broken to maximise seed germination even under adverse conditions.

The biochemical, genetic and physiological attributes of seeds are of paramount importance for the germination and survival tobacco seeds and are also critical for seed quality and agricultural yield of tobacco. Approaches of improving and stabilizing seed germination are the foundation of tobacco crop success (Gupta *et al.,* 2008) Among them seed after-ripening (Holdsworth *et al.,* 2008), imbibition in phyto-hormones (Leubner Metzger, 2012) and soaking in hydrogen peroxide (Bailly and Maarouf Bouteau, 2008) can be used. Relatively little is known about the interconnected molecular key processes regulating seed germination and dormancy in response seeds enhancement and environmental cues.

At molecular level various enzymes play a critical role in alleviating dormancy and subsequent promotion of germination of seeds such as β-1,3-glucanase, cellulose, chitinase, endo-β-mannanase, expansin, galactosidase, mannosidase, pectin methylesterase, peroxidase, polygalacturonase and xyloglucan endo-transglycosylase. (Chen and Bradford, 2000; Mo and Bewley, 2002). Of importance in tobacco is the enzyme β-1,3-glucanase in the Pathogen Related (PR) protein family which has found also to be important to crop plants like *Pisum sativum, Hordeum vulgare , Zea mays* and *Triticum aestivum* (Wu *et al.,* 2001).

Various studies have shown that enzyme β-1,3-glucanase develops prior to germination in the micropylar region of the endosperm of tobacco seeds (Leubner-Metzger *et al.,* 2006) and tomato (Morohashi and Matsushima, 2000). The enzyme contributes to the hydrolysis of 1.3 glucan linkages especially in the micropylar endosperm, which is the major constraint that prevents germination in tobacco seeds (Leubner-Metzger *et al.,* 2006). Micropylar endosperm weakening is a prerequisite for tobacco seed germination (Wu and Bradford, 2003). Fresh seeds of tobacco have non-deep physiological dormancy and germinate poorly, thereby restricting seedling establishment (Leubner-Metzger *et al.,* 2006).

Therefore, cultivation of tobacco could be easier and also more production could be reached by increasing germination of its seeds under field conditions. Little information is available on how seed after-ripening temperature and imbibition solutions affect β-1,3-glucanase activity. Thus for the local varieties (Zimbabwe), it is essential to investigate seed after-ripening temperature effects on β-1,3-glucanase activity so as the provide molecular explanation for dormancy mechanisms and germination improvement methods in tobacco. The activity of an important germination enzyme, β-1,3-glucanase, in germinating seeds was investigated to find the possible dormancy mechanism.

5.2 Main objective

1. To determine the effect of after-ripening temperature and imbibition solutions on β -1, 3-glucanase activity in KRK 26 R and T71 tobacco seeds under 30 ^{o}C light and dark.

5.2.1 Specific objectives

1. Effect of after-ripening temperature and imbibition solution on β -1, 3-glucanase activity in KRK 26 R and T71 tobacco seed under in 30 ^{o}C light.
2. Effect of after-ripening temperature and imbibition solutions on β -1, 3-glucanase activity in KRK 26 R and T71 tobacco under 30 ^{o}C dark.

5.3 Hypothesis

1. After-ripening temperature and imbibition solution has effect on β -1, 3-glucanase activity in KRK 26 R and T71 tobacco seed under 30 ^{o}C light.
2. After-ripening temperature and imbibition solutions has effect on β -1, 3-glucanase activity in KRK 26 R and T71 tobacco seed under 30 ^{o}C dark.

5.4 Materials and methods

5.4.1 Experimental Design.

The experiment followed a 2 x 4 factorial treatment structure in a completely randomized design with three replications as explained in Section 4.4.1.

After 8 weeks of storage, tobacco seeds were sampled from the two after-ripening storage temperatures and imbibed in different solutions as explained in Section 3.4 to determine β -1, 3-

glucanase for two local Kutsaga varieties (KRK 26 R and T71). For each experimental plot of 0.2 grams seeds were sown in 9-cm-diameter petri dishes incubated germination chamber with 30 ^{o}C light or dark for three days.

5.4.2 β -1, 3-glucanase activity assay protocol

After three days of incubation the seeds were removed from the growth chamber and powdered thoroughly in liquid nitrogen using a pestle in a chilled mortar. To prepare enzyme extract, seeds were crushed and homogenized in 2 ml of 15 mM sodium acetate buffer, pH 5.0 in a 2 mL micro-centrifuge tube (Eppendorf, Germany) The homogenate was centrifuged at 10000 rpm at 4 °C for 10 minutes (Morohashi and Matsushima, 2000,) in prism micro centrifuge (Labnet, USA). The β-1,3-glucanase activity was determined using the laminarin-dinitrosalicylic acid method based on the reducing sugar method of Miller (1959) but recently used by (Pan *et al.*, 1991; Ramyabharathi *et al.*, 2012) with modifications.

One hundred micro litres of 4 % (wt/vol) laminarin (Sigma) was mixed with 100 µl of the enzyme sample from the supernatant. The reaction was allowed to proceed for 10 minutes at 40 ^{o}C and stopped by addition of 600 µl of dinitrosalicylate reagent and 5 minutes of boiling (95 ^{o}C) and vortexing using a heating block (Labnet Vortemp 56, USA). Two microliters 200 µl of the mixture was placed in a micro well of the micro plate (Nuclon, Denmark) and the absorbance of the reaction was determined at 540 nm using the EZ Read 400 micro-plate reader (Biochrom, USA). To determine the reducing sugar content produced, the line equation for the glucose standard calibration curve was used. The enzyme activity was expressed µg of glucose released / min / g of sample.

5.4.3 Statistical analysis

Analysis of variance was performed on the enzyme activity data using Genstat version 17.0 and mean separation was done using the Fisher's Protected Least Significant Difference test (LSD).

5.5 Results.

5.5.1 Effect of after-ripening temperature and ambition solution on β -1, 3-glucanase activity T71 incubated in 30 ^{o}C light

In T71 imbibition solutions and after-ripening temperature had an interaction on β-1.3-glucanase activity ($P < 0.05$). There were no significant differences between the control and seeds imbibed in hydrogen peroxide regardless of the after-ripening level (Fig. 5.1.x). However benzyl adenine imbibed seeds had significantly lowest β-1.3-glucanase activity than the control regardless of the after-ripening temperature. Water + 30 ^{o}C treated seeds and GA imbibed seeds significantly increased β-1.3-glucanase activity than the control. The highest germination was attained in GA imbibed seeds though no significant differences from was attested in seeds exposed in two after-ripening temperature regimes.

5.5.2 Effect of after-ripening temperature and imbibition solution on β -1, 3-glucanase activity KRK 26 R incubated in 30 ^{o}C light.

There was an interaction between after-ripening temperature and imbibition solutions ($P < 0.05$) on β-1.3-glucanase activity of KRK 26 R light incubated seeds (Fig. 5.1.y) No significant differences were detected on β-1.3-glucanase activity between the control and seeds imbibed in hydrogen peroxide. Seeds imbibed in benzyl adenine had significantly lower activity than the control (Fig 5.1.y). Water + 30 ^{o}C treated seeds and GA imbibed seeds significantly increased β-

1.3-glucanase activity compared to the control with the highest β-1.3-glucanase activity observed in seeds after-ripened at 30 ^{o}C and imbibed in gibberellic acid.

5.5.3. Effect of after-ripening temperature and imbibition solution on β -1, 3-glucanase activity KRK 26 R incubated dark.

There was an interaction between after-ripening temperature and imbibition solutions on β-1.3-glucanase activity of KRK 26 R seeds incubated in dark ($P < 0.05$). There was no merit in imbibing seeds in hydrogen peroxide or in benzyl adenine since no stastically noticeable difference were detected between effects of this solution and the control (Fig. 5.1.z). However similar to light imbibed seeds gibberellic acid improved germination of dark imbibed seeds. Seeds after-ripened at 30 ^{o}C and imbibed in water significantly increased β-1.3-glucanase activity in KRK 26 R incubated in dark. GA imbibed seeds had significantly the highest β-1.3-glucanase activity with 30 ^{o}C after-ripened seeds recording the highest activity.

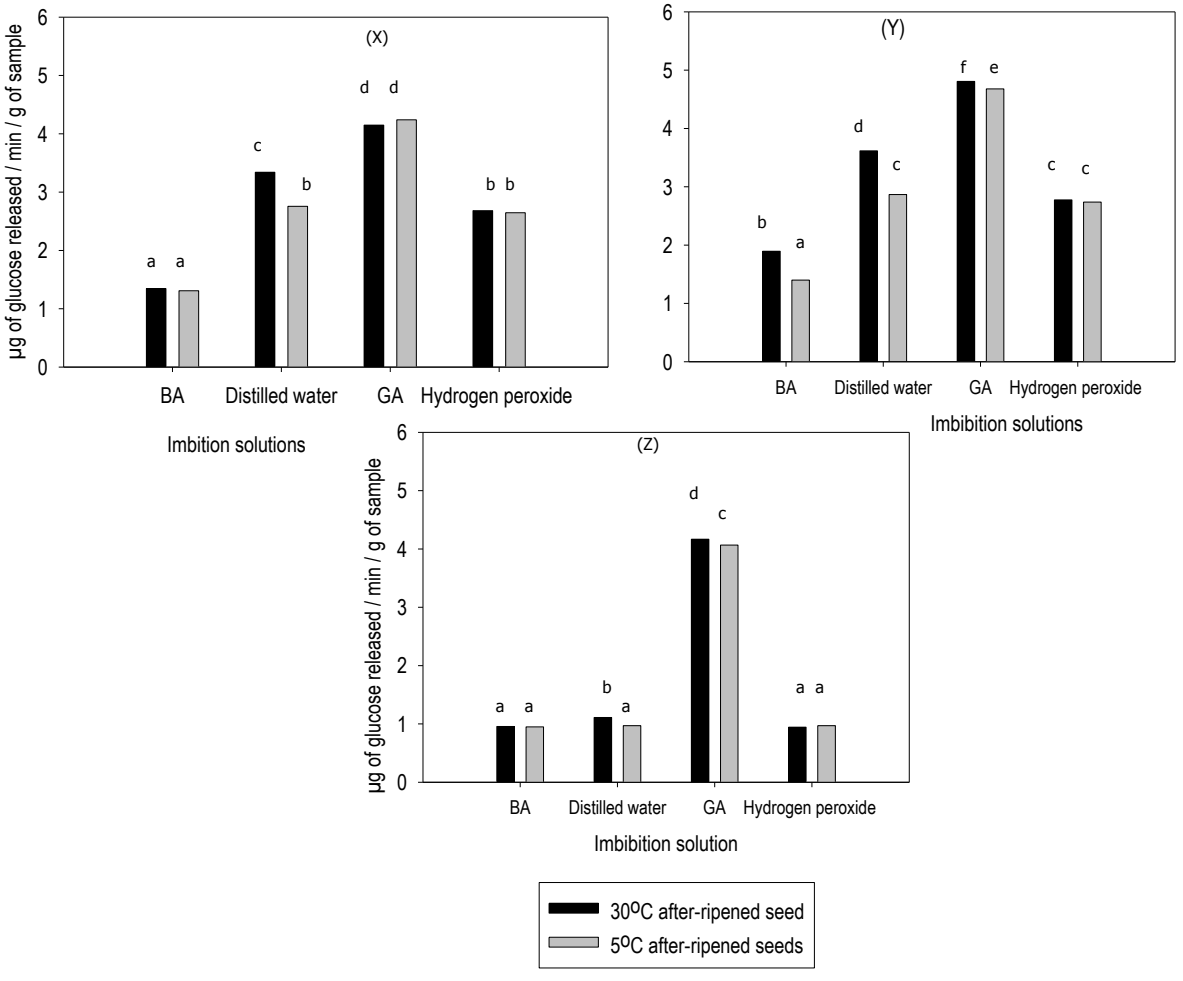

Figure 5.1. Comparison of observed mean β-1.3-glucanase activity micrograms of glucose released / min/ g of sample for seeds of T71 incubated in light (x), KRK 26 R incubated in light (y), and KRK 26 R incubated in dark (z). Bars with a common are not significantly different at 5 % level of significance.

5.5.4 Effect of after-ripening temperature and imbibition solution on β -1, 3-glucanase activity T71 incubated dark.

There was no interaction between after-ripening temperature and imbibition solution of β-1.3-glucanase in T71 seeds ($P > 0.05$). The β-1.3-glucanase activity varied significantly due to the main effecst *viz* after-ripening temperature and imbibition solution ($P < 0.05$) (Table 5.1). The β-1.3-glucanase activity was significantly improved by after-ripening seeds at 30 ^{o}C as compared to

5 ºC after-ripened seed (Table 3). The β-1.3-glucanase activity differed significantly among imbibition solutions with the highest β-1.3-glucanase activity attained in gibberellic acid imbibed seeds. BA and hydrogen peroxide significantly reduced β-1.3-glucanase activity in T71 seeds incubated in dark (Table 5.1).

Table 5.1. The β-1.3-glucanase activity (µg of glucose released / min / g of sample) of T71 seeds incubated in dark and treated with different after-ripening temperatures and imbibition solutions.

Factor	β-1.3-glucanase activity (µg of glucose released / min / g of sample).
After-ripening temperature	
5 ºC	1.687[a]
30 ºC	1.791[b]
P value	0.001
SED	0.00264
Imbibition solution	
BA	0.922[a]
Hydrogen peroxide	1.267[b]
Distilled water	1.317[c]
Gibberellic acid	3.450[d]
P value	<0.001
SED	0.0373
CV%	3.7

Means with same superscript indicate no significant differences (P< 0.05).

5.5 Discussion

The overall objective of this study was to explore the effect of after-ripening temperature and imbibition solutions on β-1,3-glucanase activity of tobacco seeds. It appears there are no previous reports on the effect of imbibition solutions and after-ripening on β-1,3-glucanase activity in tobacco seeds. The results obtained in this study demonstrated that the activity of β-1,3-glucanase was more influenced by application of exogenous application of gibberellic acid compared to other treatments in both dark imbibed seeds and light imbibed seeds.

Leubner-Metzeger (2002) outlined that the expression of β-1,3- glucanase in tobacco seeds is under the regulation of ABA which inhibits endosperm rupture through down regulation of β-1,3-glucanase expression. The β-1,3- glucanase activity is promoted by both GA and ethylene (Leubner-Metzeger, 2002). The expression of β-1,3- glucanase during germination of tobacco seeds depends on ethylene, through the combined effect of ethylene responsive elements and ethylene responsive binding proteins which are transcription factors targeted by ethylene-dependant signal transduction (Leubner-Metzger *et al.,* 1998; Petruzzelli *et al.,* 1999).

Leubner-Metzger *et al.* 2002 reported that the light / gibberellin pathway is responsible for the induction of β-1,3- glucanase with light being indirectly involved in production of endogenous GA through the phytochrome system. Temperature is a primary factor regulating tobacco seed germination and the activity of germination important enzymes such as β-1,3- glucanase activity. When tobacco seeds are exposed to high temperatures such as 30 ^{o}C, there is increase in ABA concentration and reduction in ethylene production which in turn reduces β-1,3- glucanase activity (Leubner-Metzger, 2003; Gonai *et al.,* 2004). This increased ABA concentration and increased ethylene can be an explanation why there is lower β-1,3- glucanase activity in seeds

which were not imbibed in GA. However this inhibition is alleviated by light and exogenous GA_3 which enhancing the β-1,3- glucanase activity through counteracting the effects of ABA and stimulates ethylene production (Dutta *et al.*, 1997 Jacobsen *et al.*, 2002; Leubner-Metzger, 2003).

Tavakkol *et al*. (2011) reported the same effect of exogenous gibberellic acid in their work with *Origanum vulgare* and concluded that exogenous application of GA increases β-1,3- glucanase activity. Wu *et al.* (2001) reported doubling of β-1,3-glucanase activity in the micropylar region of tomato seeds as a result of exogenous application of gibberellic acid after which occurred after 36 hours of incubation at 30ºC. This gibberellin response results are also consistent with findings of Walker-Simmons (1988) in wheat and Gonai *et al.* (2004) on endo-beta mannanase which is similar in function with β-1,3- glucanase in *Pisum sativum L.*

Increased β-1,3- glucanase activity was noted in seeds after-ripened in seeds after-ripened at 30 ºC as compared to seeds after-ripened at 5 ºC and based on these results the process of after-ripening seems to contributes to the increase in β-1,3- glucanase activity. Holdsworth *et al.*, 2008 outlined that the after-ripening process causes reduction in ABA, this to reduction in ABA concentration enhances β-1,3- glucanase expression since ABA down regulates the β-1,3- glucanase expression. Since the 30 ºC seems to fasten the after-ripening of KRK 26 R and T71 this might be the reason of increased enzyme expression in seeds after-ripened at 30 ºC. Leubner Metzger and Meins (2000) in work with Havana 425 tobacco seed also reported similar results and concluded that reduced ABA concentration by after-ripening enhances β-1,3- glucanase expression.

GA stimulated expression of β-1,3- glucanase in this experiment. In presence of light the GA and light interaction is responsible for synergistic induction of β-1,3- glucanase expression (Leubner Metzger and Meins, 2000). However in absence of light the presence of exogenous GA replaces the light requirement in induction of β-1,3- glucanase (Leubner-Metzger, 2003), this might be an

explanation why there is an increased β-1,3- glucanase activity in GA imbibed seeds and reduced activity in other treatments under dark conditions.

5.6 Conclusions and Recommendations

The findings of this study revealed that

1. Imbibition in gibberellic acid is a useful technique in enhancing β-1,3- glucanase activity thus this can be helpful in enhancing tobacco seed germination under supra optimal temperatures.
2. Based on the results of this study it can be concluded that after-ripening at 30 ^{o}C slightly enhances β-1,3- glucanase activity in seeds imbibed in hydrogen peroxide, benzyl adenine and gibberellic acid . This after-ripening temperature can therefore be used for enhancing germination of the two cultivars (KRK 26 and T7) used in this study, under supra-optimal temperature of 30^{o}C.
3. There is increased β-1,3- glucanase activity under light incubation as compared to dark incubation .

The following recommendations are made for tobacco seeds germination:

1. Imbibition in gibberellic acid can be used to enhance germination under supra optimal temperatures in the float tobacco seedling production since it is associated with increase in β-1,3- glucanase activity.
2. For conventional tobacco seedling production it is recommended that 30 ^{o}C after-ripening seeds can be used due to enhanced β-1,3- glucanase activity.
3. Farmers are recommended to place their seeds and where there is maximum light especially red light to enhance β-1,3- glucanase activity and subsequent germination .

The following recommendations are made for further work:

1. Characterization of the β-1,3- glucanase activity in local varieties and exploration of gene transfer methods and breeding methods in order to transfer genes encoding β-1,3-glucanase production from better germinating varieties to poor germinating varieties .
2. Determination of whether increased β-1,3- glucanase activity can also be beneficial in negating the effect of seed borne diseases

CHAPTER 6

6.0 EFFECT OF DRY HEAT TREATMENT AND IMBIBITION SOLUTIONS ON TOBACCO SEED GERMINATION PERFORMANCE INCUBATED UNDER 30 ^{O}C LIGHT AND 30 ^{O}C DARK.

6.1 Introduction.

Seed dormancy is an important factor in the dynamics of many natural populations and ensures that germination occurs at the most advantageous time and place. Rapid and uniform germination is a critical requirement for tobacco seedling production and methods to stimulate germination are beneficial to commercial seedling production by ensuring timely supply of the quality seedling to the grower. Dry heat treatment (DHT) has been found to be effective in breaking dormancy and stimulating rapid germination in seeds of a variety of species (Baskin and Baskin, 1998) through its physical effect on the integrity of seed coat structure (Bell *et al*., 1993) and/or physiological effect on the seed embryo (Bell and Williams, 1998).

Exposure to temperatures between 80 and 100 °C was sufficient to break the seed dormancy and stimulate germination of *Eucalypt savannas* (Williams *et al.,* 2003). Williams *et al.* (2004) reported that breaking seed dormancy is possible with temperatures below 80 °C for *Indigofera hirsuta* and 100 °C for *Galactia tenuiflora*. DHT has also been used as a dormancy breaking method in *Elaeis guineensis* (Chanprasert *et al.,* 2012) and it increased the mean germination rate of *Erica ustralis* (Vera *et al.,* 2010). Basra *et al.* (2004) also reported that exposure of *Gossypium hirsutum* seeds at 60 ^{o}C markedly improved seedling emergence and vigor. DHT has been accepted as a dormancy breaking and germination stimulation approach in tobacco (Mukarati *et al.,* 2014), however its complementary effect with other treatments to enhance germination are still necessary to achieve rapid germination with low investment.

Pragmatic alternatives include after-ripening and imbibition in various chemical solutions such gibberellic acid (GA), benzyl adenine (BA) and hydrogen peroxide. Heat treatments are mimicked in nature under various environmental conditions and climatic regions; and plant seeds sense signals on when to germinate to ensure survival. The aim of this chapter was to assess the impact of DHT stimuli on seed germination of the two tobacco varieties *viz* KRK 26 R and T71 from two after-ripening temperatures and imbibed in different imbibition solutions.

6.2 Materials and methods

6.2.1 Experimental Design

This experiment was a 4 x 4 factorial treatment structure laid out in a Completely Randomized Design with four replications. DHT conditions had four levels (5 ^{o}C after-ripening only, 30 ^{o}C after-ripening only, 5 ^{o}C after-ripening + DHT, 30 ^{o}C after-ripening + DHT) and imbibition solutions had four levels (BA, GA, hydrogen peroxide and distilled water). The control was distilled water +5 ^{o}C after-ripening.

6.2.2 DHT procedure

Half a gram seeds was sampled from the two after-ripening temperatures *viz* 5 ^{o}C and 30 ^{o}C were placed in an incubator (Heraeus Instruments GmbH, 63450 Hanau, Germany) and exposed to DHT of 37 ^{o}C for seven days. The petri dishes were sealed with parafilm (Parafilm "M"® Laboratory Film, American Can Company, Greenwich, CT, 06830) to prevent moisture loss. The seeds was were then incubated in a 30 ^{o}C germination chamber in light and dark.

6.3 Data analysis.

The seed germination percentages obtained were arcsine-square root transformed to normalize the data before statistical analysis. To distinguish significant differences among means a two way ANOVA was done on the germination percentage data from the experiments using the Genstat version 17. Means were separated by Duncan's multiple range test ($P < 0.05$).

6.4 Results

6.4.1 Effect of dry heat treatment and imbibition solutions on germination percentage of KRK 26 R tobacco seeds incubated in 30 ºC light.

Significant interaction was attested between imbibition solutions and after ripening temperature ($P < 0.05$). BA imbibed seeds displayed low germination percentage regardless of the DHT levels (Fig 6.1) DHT improved germination in all imbibition solutions except in water imbibed seeds after-ripened at 5 ^{o}C and GA imbibed seeds after-ripened at 5 ^{o}C (Fig 6.1). GA imbibed seeds showed high germination performance with the highest germination attained in seeds treated with the combination of dry heat treated seed from the 30 ^{o}C after-ripening storage. (Fig 6.1). Imbibing seeds in hydrogen peroxide also improved germination as compared to the control in three levels of DHT except for non-DHT seeds dry stored at 5 ^{o}C which showed no significance with the control .Water imbibed DHT seeds and non-DHT seeds from 30 ^{o}C after-ripening temperature also improved germination and the results were comparable with those of hydrogen peroxide imbibed seeds from the same after-ripening temperature.

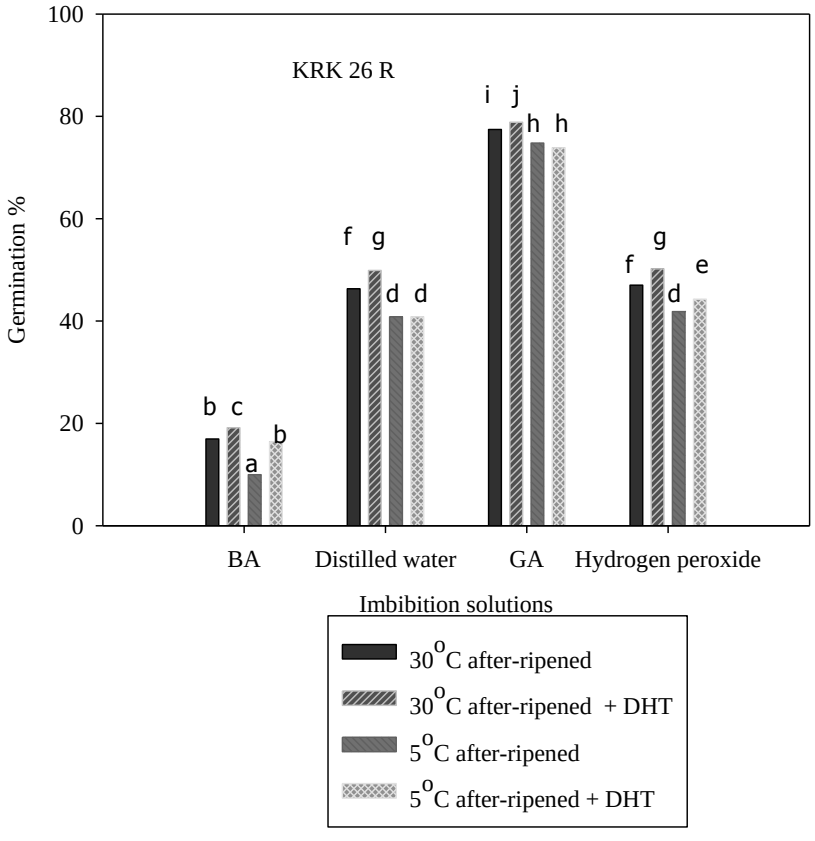

Figure 6.1. Effect of dry heat treatment and imbibition solutions on germination percentage of KRK 26 R tobacco seeds incubated under 30 ºC light. Bars with same letter not different at 5 % level of significance.

6.4.2 Effect of dry heat treatment and imbibition solutions on germination percentage of T71 tobacco seeds incubated in 30 ºC light.

The DHT x imbibition solutions interaction ($P < 0.05$) was observed on germination percentage of T71 seeds. DHT improved germination compared to non-DHT, in each of the imbibition solutions. BA imbibed seeds significantly reduced germination percentages in all DHT levels , however water imbibed seeds treated 30 ºC after-ripening only, 5 ºC after-ripened + DHT and 30 ºC after-ripening + DHT showed improved germination percentage than the control (Fig 6.2). Similar to

germination of KRK 26 R seeds, T71 seeds showed improved germination when imbibed in GA with the highest germination percentage attained in 30 ^{o}C+ DHT seeds, though not significantly different with non-DHT seeds after-ripened at 30 ^{o}C.

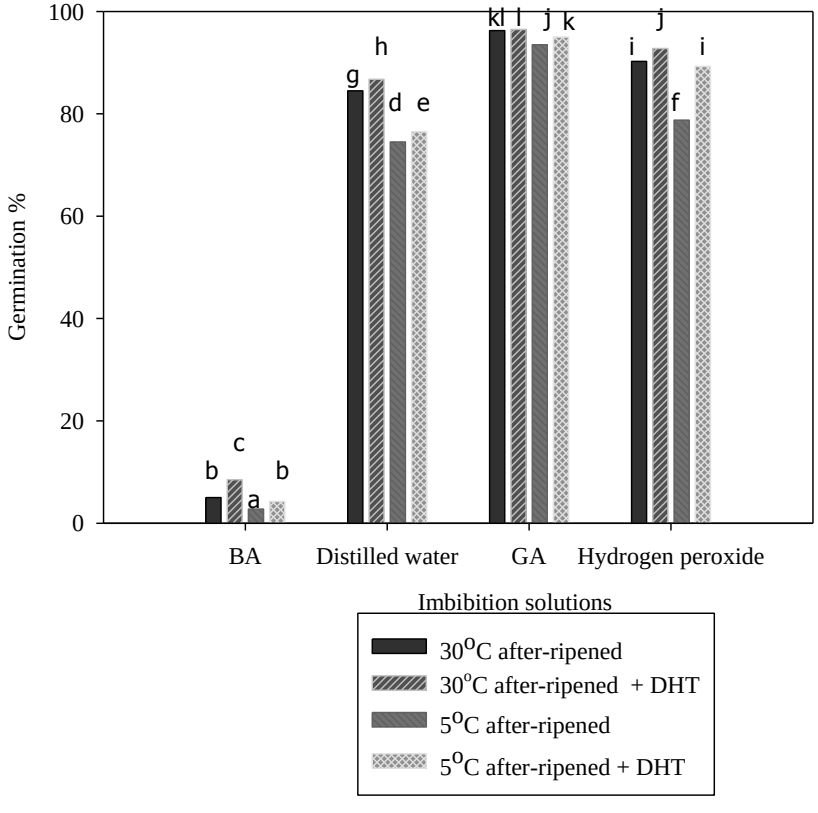

Figure 6.2. Effect of dry heat treatment and imbibition solutions on germination percentage of T71 tobacco seeds incubated under 30 ^{o}C light. Bars with same letter not different at 5 % level of significance.

6.4.3 Effect of dry heat treatment and imbibition solutions on germination percentage of KRK 26 R tobacco seeds incubated in 30 ^{o}C dark.

The interaction effect of DHT and imbibition was noted on germination percentage of dark incubated KRK 26 R seeds ($P < 0.05$). Seed after-ripened at 5 ^{o}C and imbibed either in hydrogen

peroxide or BA showed no significant differences with the control on germination percentage (Fig. 6.3). Seeds imbibed in water and either exposed to 30 ^{o}C after-ripening temperature or 5 ^{o}C after-ripening + DHT treated seeds also showed no significant difference with the control. Seeds after-ripened at 30 ^{o}C only or after-ripened at 30 ^{o}C + DHT or treated with 5 ^{o}C + DHT showed a slight increase in germination percentage when imbibed in BA or hydrogen peroxide. GA significantly increased germination performance on KRK 26 R seeds with the seeds from 30^{o}C dry storage dry heated and imbibed in GA t synergistically improved germination and attained the highest germination percentage of 73.59 (Fig. 6.3).

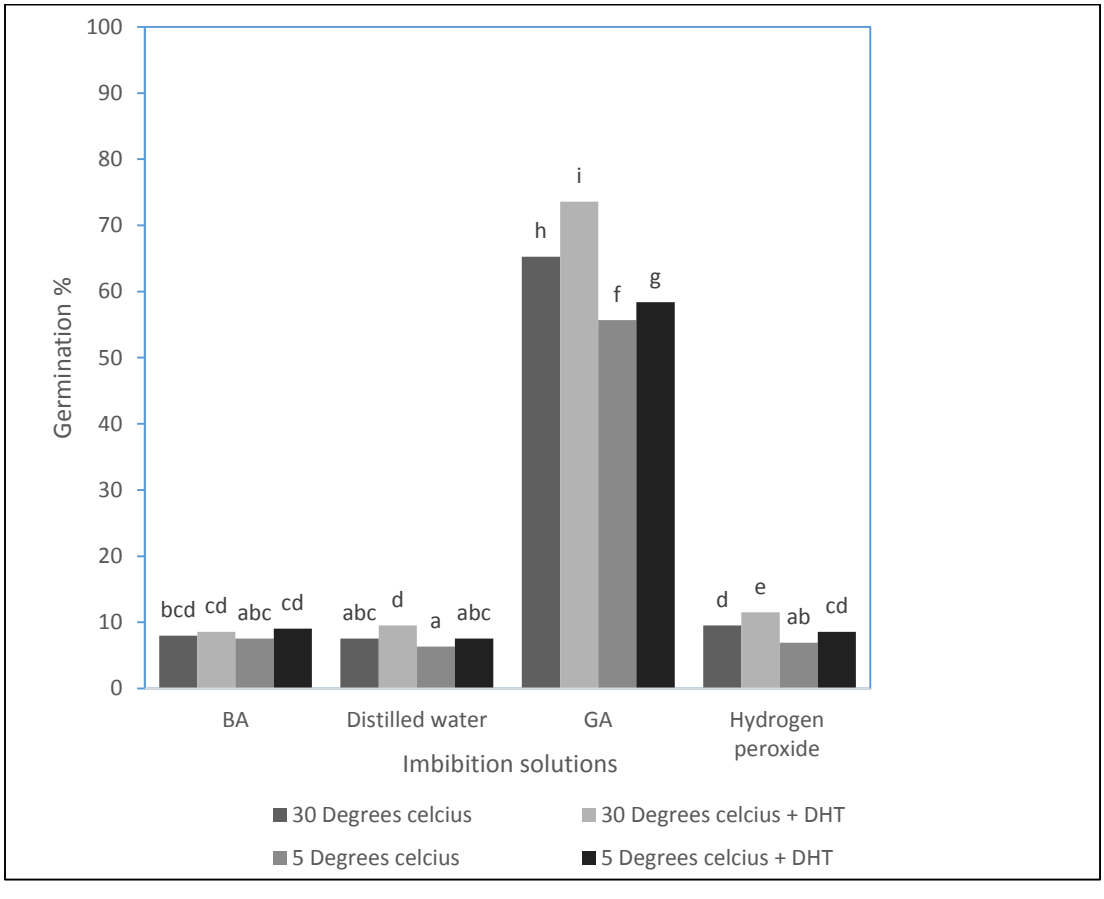

Figure 6.3. Effect of dry heat treatment and imbibition solutions on germination percentage of KRK 26 R tobacco seeds incubated under 30 oC dark Bars with same letter are not different at 5 % level of significance.

6.4.4 Effect of dry heat treatment and imbibition solutions on germination percentage of T71 tobacco seeds incubated in 30 ^{o}C dark.

DHT and imbibition solution had interaction ($P < 0.05$) on germination performance of T71 seeds under dark conditions. Exposing seeds in after-ripening temperature of 5 ^{o}C + BA imbibition reduced germination percentage with seeds after-ripened at 30 ^{o}C and imbibed in water showed a marginal increase in germination percentage (Fig 6.4). However seeds treated with a 30 ^{o}C after-ripening + DHT imbibed in hydrogen peroxide also showed marginal germination percentage increases dark incubated T71 seeds. Like in other 30 ^{o}C incubated seeds gibberellic acid imbibed seeds showed significantly higher germination percentages than the rest of the treatments.

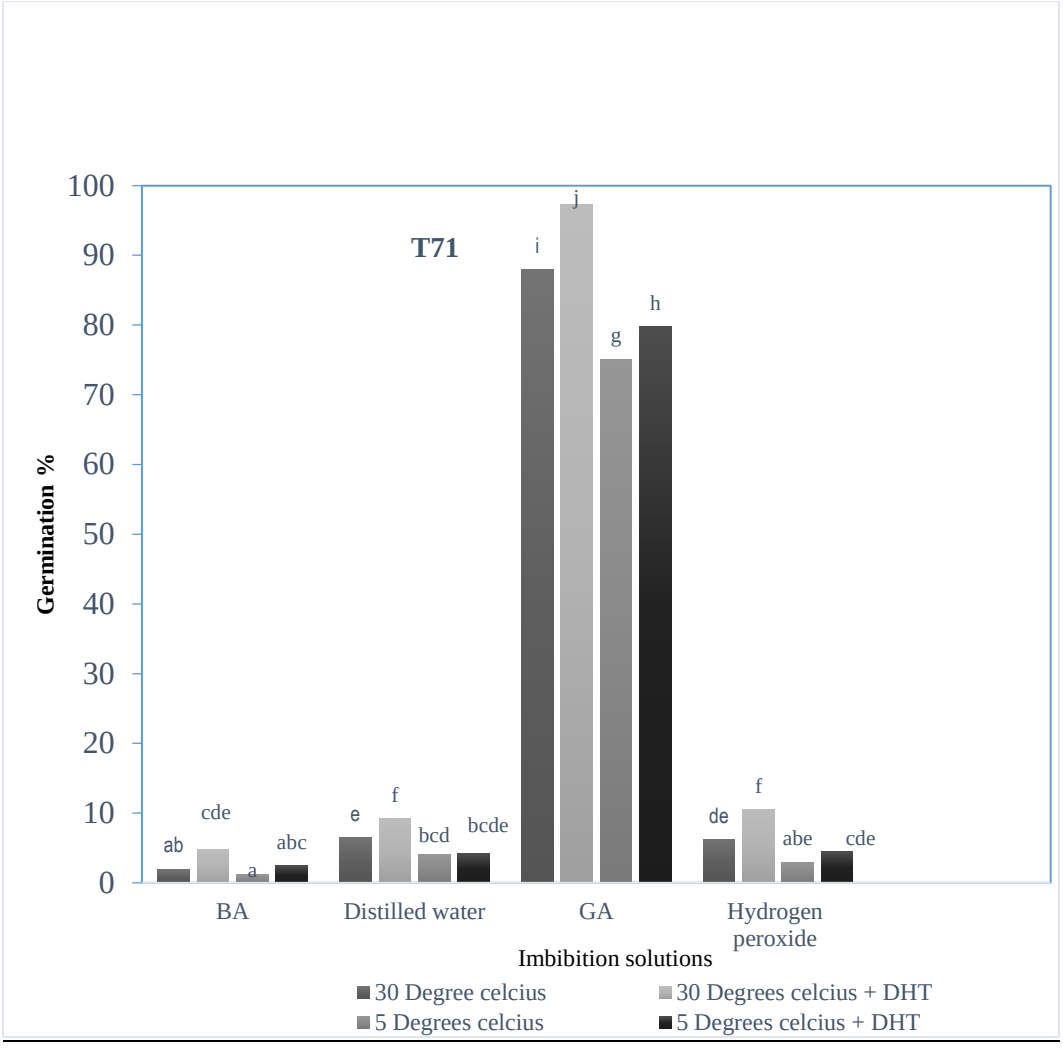

Figure 6.4. Effect of dry heat treatment and imbibition solutions on germination percentage of T71 tobacco seeds incubated under 30 ºC dark. Bars with same letter are not difrent at 5 % level of significance.

6.5 Discussion

Seed germination was not only influenced by imbibition solution but also by DHT conditions as evident by continued imbibition solution x DHT interaction throughout this experiment. The results showed significant improvement in germination percentage of DHT T71 tobacco seeds under 30 ºC light. In KRK 26 R seeds dry heat treated also showed notable improvement in

germination performance except for seeds after-ripened at 5 ^{o}C and imbibed either in distilled water or BA.

The improved germination performance due to DHT was also noted by Gashaw and Michelsen (2002) in their work with 21 plant species from savanna woodlands and grasslands of Ethiopia. In that study seeds of five species subjected to DHT temperatures of 20, 60, 90,120,150 and 200 ^{o}C showed improved germination percentage with the increase in DHT temperatures and seeds even responding to temperatures as high as 200 ^{o}C. Temperature in one critical environmental aspect which disrupts the integrity of the seed coat and thus improves the penetration of water and oxygen during seed germination (Teketay, 1998; Vera *et al,* 2010; Denton *et al.,* 2013). This might be the explanation why there is an increase in germination performance of dry heated seeds under 30 ^{o}C incubation.

These findings are also supported by the findings of Mohammadi *et al.* (2012) in okra characterized by seed hardness which showed improved germination performance when exposed to 50 and 75 ^{o}C for five minutes or exposure for a minute at 100 ^{o}C. In related experiments DHT is rather applied as a complementary strategy in enhancing seed germination, for example, in work of Nkomo and Kambizi (2009), application of a 30 ^{o}C DHT increased germination of pre-chilled *Corchorus olitorius* L. (Tiliaceae). In germination of wild species and cultivated *Oryza sativa* (rice) varieties, Waheed *et al.* (2012) noted improved germination in seeds exposed to a 50 ^{o}C dry treatment for a fortnight with a combination of dry heat treatment + dehulling synergistically influencing seed germination in *O. sativa* . In tobacco Mukarati *et al.* (2014) also noted improved germination percentages in pre-chilled seeds followed by DHT.

Exposure of plants and seeds to DHT is related to a transient increase in heat shock proteins (Devi *et al*, 1999). Evidence of heat shock proteins production noted by Devi *et al*. (1999) in pigeon pea, where exposure to 40 ^{o}C for an hour led to production of 14-110 kDA and subsequent increase in thermo tolerance during seed germination and this might be an explanation of increased germination performance in DHT seeds, observed in this study. Hanley and Lamont (2000) suggested that DHT improves seed germination through denaturing of seed coat inhibitors such as ABA.

Increase in temperature stimulates the production of reactive oxygen species (ROS) like hydrogen peroxide which contributes to endosperm weakening by tearing away of 1,3 glucan layer by the OH^{-} radical (Kokila *et al.,* 2014). Apart from endosperm weakening ROS production can stimulate seed germination through the production of antioxidants correlated with acquisition of thermo-tolerance (Maestri *et al.,* 2002; Wahid *et al,* 2007). The presence of ROS can be a reason of improved germination percentages in DHT treated seeds

6.6 Conclusion and recommendations

6.6.1. Conclusion

Imbibition solution and dry heat treatment interact during germination of KRK 26 R and T71 seeds incubated at 30 ^{o}C in light or dark

Conclusion for seeds incubated in light

DHT increases germination percentage as compared to non-DHT in T71 and KRK 26 R (except in seeds after-ripened at 5 ^{o}C and imbibed either in BA or hydrogen peroxide). Hydrogen peroxide in any combination of DHT levels increases germination percentage of T71 seeds. High

germination percentages in both KRK 26 R and T71 seeds were attained in GA imbibed seeds with the highest germination in seeds treated with 30 ^{o}C after-ripening + DHT. Distilled water imbibed seeds either after-ripened at 30 ^{o}C or 30 ^{o}C after-ripened + DHT improved germination of both KRK 26 R and T71

Conclusion for seeds incubated in dark

High germination percentages in both KRK 26 R and T71 seeds were attained in GA imbibed seeds with the highest germination in seeds treated with 30 ^{o}C after-ripening + DHT. Imbibing seeds in distilled water treated with 30 ^{o}C after-ripened seed or 30 ^{o}C after-ripened + DHT slightly improves germination in T71 tobacco seeds and in KRK 26 R seeds 30 ^{o}C after-ripening + DHT also improves germination percentage. In seeds imbibed in BA imbibed seeds treated with 30 ^{o}C after-ripening, 30 ^{o}C after-ripening + DHT, 5 ^{o}C after-ripening + DHT slightly increased germination percentage of KRK 26 R seeds. Imbibing seeds in hydrogen peroxide treated with 30 ^{o}C after-ripening or 30 ^{o}C after-ripening +DHT or 5 ^{o}C after-ripening + DHT slightly increased germination percentage of KRK 26 R seeds

6.6.2. Recommendations

Based on the results it is recommended that 30 ^{o}C after-ripening + DHT followed by imbibition in GA can be used as germination treatment to enhance germination under laboratory conditions whether in light or dark. For field tobacco seedling production the 30 ^{o}C after-ripened seed + DHT can be used. Further work need to be explored on how to incorporate GA in field tobacco seedling production.

CHAPTER 7

7.0 GENERAL DISCUSSIONS, CONCLUSIONS AND RECOMMENDATIONS

7.1 General discussions

Throughout this study it is evident that germination of tobacco seeds depends on incubation temperature and light. However, the germination responses were different across the germination environments with the best germination performance noted in seeds incubated in 20 ^{o}C light compared to other germination environments. This buttresses the findings of Hartley *et al*. (2001) who concluded that the germination of tobacco is best under a temperature range of 18-23 ^{o}C.

Under 30 ^{o}C a reduction germination percentage was noted in KRK 26 R and T71. This might be due to the effect of thermo-inhibition and subsequent thermo-dormancy (Gonai *et al*., 2004). This reduction in germination performance under this incubation temperature can be attributed to increase in ABA and ethylene as both ethylene and ABA influence the germination process. The increased ABA concentration and reduced ethylene production down regulate the β-1,3-glucanase induction leading to reduced endosperm weakening as postulated by Leubner Metzger (2002); Leubner-Metzger *et al.* (1998); Petruzzelli *et al.* (1999).

Based on these results it is also evident that at 30 ^{o}C there is reduction in ß -1,3 glucanase activity which might be the reason for reduction in germination performance which is also supported by the findings of Tavakkol *et al.* (2011) on their work with *Origanum vulgare.* The enzyme, β-1,3-glucanase plays an indirect role as a signalling molecule to mediate loosening or breakage of the micropylar endosperm by releasing elicitor- active oligo-ß -1,3- glucans from the micropylar endosperm components (Tavakkol, *et al.*, 2011).

Apart from temperature, it is evident from this study, that the germination of tobacco seeds is depended on light. In small seeded crop species light is a major environmental factor that influences germination (Milberg *et al.,* 2000; Yamaguchi and Kamiya, 2002). In presence of red light there is an increase in mRNA levels of GA 3-oxidase which catalyses the production of GA through the conversion of phytochrome red to phytochrome far-red (Yamaguchi and Kamiya, 2002). The increased GA enhances induction of 1,3-glucanase and stimulates embryo growth which subsequently alleviates dormancy and enhances germination (Yamaguchi and Kamiya, 2002). However presence of dark or far red light reverses the red light–phytochrome germination stimulation pathway leading to inability of the seeds to germinate. This is the most probable explanation for reduced germination under dark conditions (Sanchez and Mella, 2004; Leubner-Metzger 2006).

In this study it is evident that the after-ripening alleviates dormancy. It is evident that the increase in after-ripening duration increases the sensitivity of tobacco seeds to GA and widens the temperature range of the germination process. The widening of the temperature range is evident and beneficial for 30 ^{o}C light incubated seed where germination of water imbibed and hydrogen peroxide imbibed seeds showed increase in germination. GA imbibed seeds also followed the same trend in which the response of seeds to GA increased with increase in after-ripening duration in most situation temperature. These results are in consistent with the reports of (Probert, 2000; Leubner-Metzger, 2003; Kucera *et al*., 2005; Bair *et al*., 2006; Leubner-Metzger 2006) who outlined that after-ripening is characterised by widening of the temperature range and increase in sensitivity of seeds to exogenous GA.

7.2 General conclusion.

7.2.1 Effect of after-ripening temperature and imbibition solution on tobacco seed germination performance under different germination environments.

After-ripening temperature and imbibition solution affect tobacco seed germination performance under different germination environments with the best germination performance attained in 20 ^{o}C light incubation. Under 20 ^{o}C light incubation seeds after-ripened at 30 ^{o}C and imbibed in either water or hydrogen peroxide give the highest germination rate. However under these conditions it can be concluded that there is no merit in imbibing seeds in phyto-hormones since low germination percentages than the control were noted. There is an overall reduction in germination percentage under 30 ^{o}C light and it can be concluded that germination can be improved by imbibition in GA and reduced by imbibition in BA. It can also be concluded that after-ripening seeds at 30 ^{o}C improves germination under 30 ^{o}C light regardless of the imbibition solutions.

Germination of KRK 26 R and T71 seeds is highly dependent on light with high germination performance noted in light imbibed seeds. Improvement of germination can be achieved by imbibition in GA. However, germination in dark can also be improved by after-ripening temperature of 30 ^{o}C as compared to 5 ^{o}C.

7.2.2 Effect of after-ripening storage temperature and imbibition solutions on β-1, 3-glucanase activity in tobacco seeds under 30 ^{o}C light.

The β-1, 3-glucanase activity is influenced by light with high activity noted in light incubated seeds as compared to dark incubated seeds. Imbibition of KRK 26 R seeds in GA enhances β-1,3-glucanase activity and after-ripening temperature slightly improves β-1,3-glucanase activity so it

can be a useful tool to enhance germination . It can also be concluded that BA imbibition reduces β-1,3-glucanase activity . Based on the results of this study it can be concluded that after-ripening at 30 ºC slightly enhances β-1,3- glucanase activity regardless of imbibition solution.

7.2.3 Effect of dry heat treatment and imbibition solution on tobacco seed germination performance under 30 ºC light and 30 ºC dark.

It can be concluded that GA, after-ripening temperature and DHT enhances germination performance under 30 ºC light and dark. The combination of after-ripening at 30 ºC followed by DHT and GA imbibition is the best combination that enhances germination under these conditions.

7. 3 General recommendations

7.3.1 Recommendations for tobacco seedling production

It is recommended that germination of KRK 26 R and T71 seeds be done at 20 ºC light conditions. It is also recommended that seeds be after-ripened at 30 ºC and be imbibed in water and hydrogen peroxide can be used to enhance germination under 20 ºC light conditions.

Is recommended that germination can be enhanced by 30 ºC after-ripening and imbibition in gibberellic acid +DHT should be used to enhance germination under supra optimal conditions IN float tray system. However in conventional tobacco seedling production it is recommended than after-ripening seeds at 30 ºC + DHT or 30 ºC can be used to enhance germination

Farmers are advised to place their seeds where they can receive maximum light and where possible red light should be used to maximise germinations in field and propagating structures. Under laboratory conditions and float systems addition of GA is recommended to circumvent the ravaging effect of dark conditions and far red light.

7.3.2 Recommendations for further research work

1. Explorations of a wider range of after-ripening temperatures
2. Exploration of other strategies be used to enhance germination under supra optimal temperatures apart from after-ripening and DHT
3. Determination of the optimal after-ripening duration under a wider range of temperature
4. Determination of a temperature after-ripening model for local tobacco varieties.
5. Investigate why phyto-hormones especially are failing to provide consistent results in tobacco seed germination under 20 ^{o}C light

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APPENDICES

Appendix 1. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination rate of T71 tobacco seeds incubated for ten days in 20 ºC light. (2 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	0.00090324	0.00030108	3.90	
Replication.*Units* stratum					
After-ripening	1	0.01379364	0.01379364	178.64	<.001
Imbibition	3	1.37883599	0.45961200	5952.39	<.001
After-ripening_Imbibition	3	0.00875286	0.00291762	37.79	<.001
Residual	21	0.00162151	0.00007721		
Total	31	1.40390724			

- ✓ SED 0.00621
- ✓ LSD 0.01292
- ✓ CV% 2.2

Appendix 2. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination rate of T71 tobacco seeds incubated for ten days in 20 ºC light. (4 WAS)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	0.00027080	0.00009027	1.36	
Replication.*Units* stratum					
After-ripening	1	0.00353581	0.00353581	53.26	<.001
Imbibition	3	1.74319921	0.58106640	8753.43	<.001
After-ripening_Imbibition	3	0.00094028	0.00031343	4.72	0.011
Residual	21	0.00139401	0.00006638		
Total	31	1.74934010			

- ✓ SED 0.00576
- ✓ LSD 0.01198
- ✓ CV% 2.0

Appendix 3. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination rate of T71 tobacco seeds incubated for ten days in 20 ºC light. (6 WAS)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	0.0002936	0.0000979	0.90	
Replication.*Units* stratum					
After-ripening	1	0.0038613	0.0038613	35.47	<.001
Imbibition	3	2.4183137	0.8061046	7405.75	<.001
After-ripening.Imbibition	3	0.0038171	0.0012724	11.69	<.001
Residual	21	0.0022858	0.0001088		
Total	31	2.4285716			

- ✓ SED 0.00738
- ✓ LSD 0.01534
- ✓ CV% 2.1

Appendix 4. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination rate of T71 tobacco seeds incubated for ten days in 20 oC light. (8 WAS)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	0.00027229	0.00009076	1.26	
Replication.*Units* stratum					
After-ripening	1	0.00589046	0.00589046	81.49	<.001
Imbibition	3	2.50907100	0.83635700	11570.98	<.001
After-ripening.Imbibition	3	0.00440344	0.00146781	20.31	<.001
Residual	21	0.00151789	0.00007228		
Total	31	2.52115508			

- ✓ SED 0.00601
- ✓ LSD 0.01250
- ✓ CV% 1.7

Appendix 5. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination rate of KRK 26 R tobacco seeds incubated for ten days in 20 ºC light (2 WAS)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	0.0012390	0.0004130	3.90	
Replication.*Units* stratum					
After-ripening	1	0.0135190	0.0135190	127.80	<.001
Imbibition	3	1.7496118	0.5832039	5513.10	<.001
After-ripening.Imbibition	3	0.0096021	0.0032007	30.26	<.001
Residual	21	0.0022215	0.0001058		
Total	31	1.7761934			

- ✓ SED 0.00727
- ✓ LSD 0.01512
- ✓ CV% 2.4

Appendix 6. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination rate of KRK 26 R tobacco seeds incubated for ten days in 20 ºC light (4 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	0.00026623	0.00008874	1.22	
Replication.*Units* stratum					
After-ripening	1	0.00353581	0.00353581	48.80	<.001
Imbibition	3	1.74092319	0.58030773	8008.92	<.001
After-ripening.Imbibition	3	0.00088553	0.00029518	4.07	0.020
Residual	21	0.00152161	0.00007246		
Total	31	1.74713238			

- ✓ SED 0.00602
- ✓ LSD 0.01252
- ✓ CV% 2.1

Appendix 7. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination rate of KRK 26 R tobacco seeds incubated for ten days in 20 ºC light (6 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	0.00000632	0.00000211	0.04	
Replication.*Units* stratum					
After-ripening	1	0.00983128	0.00983128	166.40	<.001
Imbibition	3	2.56615722	0.85538574	14477.97	<.001
After-ripening.Imbibition	3	0.00220990	0.00073663	12.47	<.001
Residual	21	0.00124072	0.00005908		
Total	31	2.57944544			

- ✓ SED 0.00544
- ✓ LSD 0.01130
- ✓ CV% 1.5

Appendix 8. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination rate of KRK 26 R tobacco seeds incubated for ten days in 20 ºC light (8 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	0.00008332	0.00002777	0.96	
Replication.*Units* stratum					
After-ripening	1	0.00773298	0.00773298	267.79	<.001
Imbibition	3	2.62563390	0.87521130	30307.83	<.001
After-ripening.Imbibition	3	0.00268471	0.00089490	30.99	<.001
Residual	21	0.00060643	0.00002888		
Total	31	2.63674135			

- ✓ SED 0.003800
- ✓ LSD 0.007902
- ✓ CV% 1.1

Appendix 9. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination percentage of T71 tobacco seeds incubated for ten days in 30 ºC light (2 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	4.358	1.453	1.15	
Replication.*Units* stratum					
After-ripening	1	267.733	267.733	211.12	<.001
Imbibition	3	15720.681	5240.227	4132.19	<.001
After-ripening.Imbibition	3	73.759	24.586	19.39	<.001
Residual	21	26.631	1.268		
Total	31	16093.163			

- ✓ SED 0.796
- ✓ LSD 1.656
- ✓ CV% 2.3

Appendix 10. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination percentage of T71 tobacco seeds incubated for ten days in 30 ºC light (4 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	4.1193	1.3731	1.46	
Replication.*Units* stratum					
After-ripening	1	200.7253	200.7253	213.68	<.001
Imbibition	3	15232.2749	5077.4250	5405.14	<.001
After-ripening.Imbibition	3	15.2247	5.0749	5.40	0.006
Residual	21	19.7268	0.9394		
Total	31	15472.0710			

- ✓ SED 0.685
- ✓ LSD 1.425
- ✓ CV% 2.0

Appendix 11. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination percentage of T71 tobacco seeds incubated for ten days in 30 ºC light (6 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	5.074	1.691	0.58	
Replication.*Units* stratum					
After-ripening	1	163.288	163.288	56.44	<.001
Imbibition	3	14949.818	4983.273	1722.39	<.001
After-ripening.Imbibition	3	18.706	6.235	2.16	0.012
Residual	21	60.758	2.893		
Total	31	15197.644			

- ✓ SED 1.203
- ✓ LSD 2.501
- ✓ CV% 3.4

Appendix 12. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination percentage of T71 tobacco seeds incubated for ten days in 30 ºC light (8 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	31.961	10.654	1.54	
Replication.*Units* stratum					
After-ripening	1	518.036	518.036	74.96	<.001
Imbibition	3	19050.181	6350.060	918.84	<.001
After-ripening.Imbibition	3	229.154	76.385	11.05	<.001
Residual	21	145.131	6.911		
Total	31	19974.462			

- ✓ SED 1.859
- ✓ LSD 3.866
- ✓ CV% 4.8

Appendix 13. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination percentage of KRK26 R tobacco seeds incubated for ten days in 30 ºC light (2 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	8.563	2.854	1.91	
Replication.*Units* stratum					
After-ripening	1	214.550	214.550	143.21	<.001
Imbibition	3	14097.804	4699.268	3136.75	<.001
After-ripening.Imbibition	3	14.888	4.963	3.31	0.040
Residual	21	31.461	1.498		
Total	31	14367.265			

- ✓ SED 0.865
- ✓ LSD 1.800
- ✓ CV% 3.0

Appendix 14. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination percentage of KRK26 R tobacco seeds incubated for ten days in 30 ºC light (4 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	7.8528	2.6176	2.73	
Replication.*Units* stratum					
After-ripening	1	157.6391	157.6391	164.39	<.001
Imbibition	3	14293.2996	4764.4332	4968.42	<.001
After-ripening.Imbibition	3	16.3596	5.4532	5.69	0.005
Residual	21	20.1378	0.9589		
Total	31	14495.2890			

- ✓ SED 0.692
- ✓ LSD 1.440
- ✓ CV% 2.3

Appendix 15. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination percentage of KRK26 R tobacco seeds incubated for ten days in 30 ºC light (6 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	3.397	1.132	0.83	
Replication.*Units* stratum					
After-ripening	1	352.270	352.270	259.76	<.001
Imbibition	3	14703.555	4901.185	3614.06	<.001
After-ripening.Imbibition	3	57.719	19.240	14.19	<.001
Residual	21	28.479	1.356		
Total	31	15145.419			

- ✓ SED 0.823
- ✓ LSD 1.712
- ✓ CV% 2.6

Appendix 16. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination percentage of KRK26 R tobacco seeds incubated for ten days in 30 ºC light (8 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	9.786	3.262	1.17	
Replication.*Units* stratum					
After-ripening	1	791.208	791.208	283.42	<.001
Imbibition	3	18287.892	6095.964	2183.65	<.001
After-ripening.Imbibition	3	318.048	106.016	37.98	<.001
Residual	21	58.624	2.792		
Total	31	19465.559			

- ✓ SED 1.181
- ✓ LSD 2.457
- ✓ CV% 3.0

Appendix 17. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination percentage of T71 tobacco seeds incubated for ten days in 20 ºC dark (2 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	4.595	1.532	0.75	
Replication.*Units* stratum					
After-ripening	1	179.324	179.324	88.11	<.001
Imbibition	3	17706.287	5902.096	2899.94	<.001
After-ripening.Imbibition	3	34.322	11.441	5.62	0.005
Residual	21	42.740	2.035		
Total	31	17967.268			

- ✓ SED 1.009
- ✓ LSD 2.098
- ✓ CV% 6.8

Appendix 18. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination percentage of T71 tobacco seeds incubated for ten days in 20 ºC dark (4 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	6.851	2.284	0.56	
Replication.*Units* stratum					
After-ripening	1	189.720	189.720	46.78	<.001
Imbibition	3	18468.379	6156.126	1518.07	<.001
After-ripening.Imbibition	3	57.962	19.321	4.76	0.011
Residual	21	85.160	4.055		
Total	31	18808.072			

- ✓ SED 1.424
- ✓ LSD 2.961
- ✓ CV% 9.8

Appendix 19. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination percentage of T71 tobacco seeds incubated for ten days in 20 ºC dark (6 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	1.949	0.650	0.33	
Replication.*Units* stratum					
After-ripening	1	165.986	165.986	85.02	<.001
Imbibition	3	12535.553	4178.518	2140.31	<.001
After-ripening.Imbibition	3	8.515	2.838	1.45	0.256
Residual	21	40.998	1.952		
Total	31	12753.002			

- ✓ SED after-ripening 0.494
- ✓ SED imbibition solutions 0.699
- ✓ LSD after-ripening 1.027
- ✓ LSD imbibition solutions 1.453
- ✓ CV% 5.0

Appendix 20. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination percentage of T71 tobacco seeds incubated for ten days in 20 ºC dark (8 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	2.180	0.727	0.53	
Replication.*Units* stratum					
After-ripening	1	172.466	172.466	125.31	<.001
Imbibition	3	15499.302	5166.434	3753.93	<.001
After-ripening.Imbibition	3	3.889	1.296	0.94	0.438
Residual	21	28.902	1.376		
Total	31	15706.739			

- ✓ SED after-ripening 0.415
- ✓ SED imbibition solutions 0.587
- ✓ LSD after-ripening 0.863
- ✓ LSD imbibition solutions 1.220
- ✓ CV% 3.9

Appendix 21. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination percentage of KRK 26 R tobacco seeds incubated for ten days in 20 ºC dark (2 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	19.722	6.574	1.86	
Replication.*Units* stratum					
After-ripening	1	292.946	292.946	82.81	<.001
Imbibition	3	18006.842	6002.281	1696.82	<.001
After-ripening.Imbibition	3	14.764	4.921	1.39	0.273
Residual	21	74.285	3.537		
Total	31	18408.559			

- ✓ SED after-ripening 0.665
- ✓ SED imbibition solutions 0.940
- ✓ LSD after-ripening 1.383
- ✓ LSD imbibition solutions 1.956
- ✓ CV% 8.9

Appendix 22. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination percentage of KRK 26 R tobacco seeds incubated for ten days in 20 ºC dark (4 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	9.403	3.134	0.92	
Replication.*Units* stratum					
After-ripening	1	106.530	106.530	31.27	<.001
Imbibition	3	20621.882	6873.961	2017.98	<.001
After-ripening.Imbibition	3	21.575	7.192	2.11	0.129
Residual	21	71.534	3.406		
Total	31	20830.924			

- ✓ SED after-ripening 0.653
- ✓ SED imbibition solutions 0.923
- ✓ LSD after-ripening 1.357
- ✓ LSD imbibition solutions 1.919
- ✓ CV% 8.6

Appendix 23. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination percentage of KRK 26 R tobacco seeds incubated for ten days in 20 ºC dark (6 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	8.297	2.766	1.99	
Replication.*Units* stratum					
After-ripening	1	563.948	563.948	406.50	<.001
Imbibition	3	17720.838	5906.946	4257.79	<.001
After-ripening.Imbibition	3	25.008	8.336	6.01	0.004
Residual	21	29.134	1.387		
Total	31	18347.224			

- ✓ SED 0.833
- ✓ LSD 1.732
- ✓ CV% 4.1

Appendix 24. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination percentage of KRK 26 R tobacco seeds incubated for ten days in 20 oC dark (8 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	2.1518	0.7173	0.92	
Replication.*Units* stratum					
After-ripening	1	544.7707	544.7707	696.81	<.001
Imbibition	3	18749.1125	6249.7042	7993.95	<.001
After-ripening.Imbibition	3	86.1498	28.7166	36.73	<.001
Residual	21	16.4179	0.7818		
Total	31	19398.6027			

- ✓ SED 0.625
- ✓ LSD 1.300
- ✓ CV% 3.1

Appendix 25. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination percentage of T71 tobacco seeds incubated for ten days in 30 ºC dark (2 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	5.781	1.927	0.48	
Replication.*Units* stratum					
After-ripening	1	222.921	222.921	55.62	<.001
Imbibition	3	7724.145	2574.715	642.37	<.001
After-ripening.Imbibition	3	259.329	86.443	21.57	<.001
Residual	21	84.171	4.008		
Total	31	8296.346			

- ✓ SED 1.416
- ✓ LSD 2.944
- ✓ CV % 11.9

Appendix 26. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination percentage of T71 tobacco seeds incubated for ten days in 30 ºC dark (4 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	5.264	1.755	1.63	
Replication.*Units* stratum					
After-ripening	1	233.877	233.877	216.71	<.001
Imbibition	3	7878.379	2626.126	2433.36	<.001
After-ripening.Imbibition	3	250.677	83.559	77.43	<.001
Residual	21	22.664	1.079		
Total	31	8390.861			

- ✓ SED 0.735
- ✓ LSD 1.528
- ✓ CV% 5.9

Appendix 27. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination percentage of T71 tobacco seeds incubated for ten days in 30 ºC dark (6 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	0.245	0.082	0.08	
Replication.*Units* stratum					
After-ripening	1	137.285	137.285	131.16	<.001
Imbibition	3	14479.210	4826.403	4610.90	<.001
After-ripening.Imbibition	3	48.562	16.187	15.46	<.001
Residual	21	21.981	1.047		
Total	31	14687.283			

- ✓ SED 0.723
- ✓ LSD 1.504
- ✓ CV% 4.1

Appendix 28. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination percentage of T71 tobacco seeds incubated for ten days in 30 ºC dark (8 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	2.439	0.813	0.57	
Replication.*Units* stratum					
After-ripening	1	114.500	114.500	79.72	<.001
Imbibition	3	16731.010	5577.003	3883.02	<.001
After-ripening.Imbibition	3	26.477	8.826	6.15	0.004
Residual	21	30.161	1.436		
Total	31	16904.589			

- ✓ SED 0.847
- ✓ LSD 1.762
- ✓ CV% 4.7

Appendix 29. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination percentage of KRK 26 R tobacco seeds incubated for ten days in 30 ºC dark (2 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	18.620	6.207	1.78	
Replication.*Units* stratum					
After-ripening	1	108.036	108.036	30.91	<.001
Imbibition	3	11606.422	3868.807	1106.73	<.001
After-ripening.Imbibition	3	4.847	1.616	0.46	0.007
Residual	21	73.410	3.496		
Total	31	11811.335			

- ✓ SED 1.322
- ✓ LSD 2.749
- ✓ CV% 10.2

Appendix 30. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination percentage of KRK 26 R tobacco seeds incubated for ten days in 30 ºC dark (4 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	10.786	3.595	2.89	
Replication.*Units* stratum					
After-ripening	1	73.565	73.565	59.13	<.001
Imbibition	3	13744.812	4581.604	3682.27	<.001
After-ripening.Imbibition	3	13.081	4.360	3.50	0.033
Residual	21	26.129	1.244		
Total	31	13868.373			

- ✓ SED 0.789
- ✓ LSD 1.640
- ✓ CV% 5.9

Appendix 31. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination percentage of KRK 26 R tobacco seeds incubated for ten days in 30 ºC dark (6 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	7.612	2.537	1.42	
Replication.*Units* stratum					
After-ripening	1	136.575	136.575	76.37	<.001
Imbibition	3	17151.231	5717.077	3196.74	<.001
After-ripening.Imbibition	3	90.667	30.222	16.90	<.001
Residual	21	37.557	1.788		
Total	31	17423.642			

- ✓ SED 0.946
- ✓ LSD 1.967
- ✓ CV% 6.6

Appendix 32. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on germination percentage of KRK 26 R tobacco seeds incubated for ten days in 30 ºC dark (8 WAS).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	2.0602	0.6867	0.72	
Replication.*Units* stratum					
After-ripening	1	168.4863	168.4863	177.55	<.001
Imbibition	3	16994.7892	5664.9297	5969.72	<.001
After-ripening.Imbibition	3	10.4037	3.4679	3.65	0.029
Residual	21	19.9278	0.9489		
Total	31	17195.6672			

- ✓ SED 0.689
- ✓ LSD 1.432
- ✓ CV% 4.4

Appendix 33. Analysis of variance: Effect of after-ripening temperature and imbibition solution on β -1, 3-glucanase activity T71 incubated in 30 ºC light.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
After-ripening	1	0.14541	0.14541	13.44	0.002
Imbibition	3	25.22894	8.40965	777.38	<.001
After-ripening.Imbibition	3	0.48756	0.16252	15.02	<.001
Residual	16	0.17309	0.01082		
Total	23	26.03500			

- ✓ SED 0.0849
- ✓ LSD 0.1800
- ✓ CV% 3.7

Appendix 34. Analysis of variance: Effect of after-ripening temperature and imbibition solutions on β -1, 3-glucanase activity KRK 26 R incubated in 30 ºC light

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
After-ripening	1	0.745809	0.745809	164.69	<.001
Imbibition	3	29.680770	9.893590	2184.77	<.001
After-ripening.Imbibition	3	0.493474	0.164491	36.32	<.001
Residual	16	0.072455	0.004528		
Total	23	30.992508			

- ✓ SED 0.0549
- ✓ LSD 0.1165
- ✓ CV% 2.2

Appendix 35. Analysis of variance: Effect of after-ripening and imbibition solution on β -1, 3-glucanase activity T71 incubated in 30 oC dark.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
After-ripening	1	0.065104	0.065104	15.56	0.001
Imbibition	3	23.982112	7.994038	1910.93	<.001
After-ripening.Imbibition	3	0.025313	0.008438	2.02	0.152
Residual	16	0.066933	0.004183		
Total	23	24.139462			

- ✓ SED after-ripening 0.0264
- ✓ SED imbibition solutions 0.0373
- ✓ LSD after-ripening 0.0560
- ✓ LSD imbibition solutions 0.0792
- ✓ CV% 3.7

Appendix 36. Analysis of variance: Effect of after-ripening and imbibition solution on β -1, 3-glucanase activity KRK 26 R incubated 30 ºC dark.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
After-ripening	1	0.018150	0.018150	8.57	0.010
Imbibition	3	44.208933	14.736311	6962.04	<.001
After-ripening.Imbibition	3	0.027383	0.009128	4.31	0.021
Residual	16	0.033867	0.002117		
Total	23	44.288333			

- ✓ SED 0.03756
- ✓ LSD 0.07963
- ✓ CV% 2.6

Appendix 37. Analysis of variance: Effect of dry heat treatment and imbibition solutions on germination percentage of KRK 26 R tobacco seeds incubated in 30 ºC light

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	0.1904	0.0635	0.10	
Replication.*Units* stratum					
DHT	3	546.5210	182.1737	273.69	<.001
Imbibition	3	29411.4718	9803.8239	14728.68	<.001
DHT.Imbibition	9	95.8797	10.6533	16.00	<.001
Residual	45	29.9533	0.6656		
Total	63	30084.0162			

- ✓ SED 0.577
- ✓ LSD 1.162
- ✓ CV% 1.8

Appendix 38. Analysis of variance: Effect of dry heat treatment and imbibition solutions on germination percentage of T71 tobacco seeds incubated in 30 ºC light

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	0.1904	0.0635	0.10	
DHT	3	685.2500	228.4167	288.53	<.001
Imbibition	3	83911.6250	27970.5417	35331.21	<.001
DHT.Imbibition	9	294.8750	32.7639	41.39	<.001
Residual	48	38.0000	0.7917		
Total	63	84929.7500			

- ✓ SED 0.629
- ✓ LSD 1.265
- ✓ CV% 4.3

Appendix 39. Analysis of variance Effect of dry heat treatment and imbibition solutions on germination percentage of KRK 26 R tobacco seeds incubated in 30 ºC dark.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	3	7.0420	2.3473	2.52	
Replication.*Units* stratum					
DHT	3	388.4329	129.4776	138.81	<.001
Imbibition	3	36112.5536	12037.5179	12905.08	<.001
DHT.Imbibition	9	448.4256	49.8251	53.42	<.001
Residual	45	41.9748	0.9328		
Total	63	36998.4289			

- ✓ SED 0.683
- ✓ LSD 1.375
- ✓ CV 4.4

Appendix 40. Analysis of variance: Effect of dry heat treatment and imbibition solutions on germination percentage of T71 tobacco seeds incubated in 30 ºC dark.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Imbibition	3	77244.125	25748.042	11235.51	<.001
DHT	3	833.625	277.875	121.25	<.001
Imbibition.DHT	9	518.000	57.556	25.12	<.001
Residual	48	110.000	2.292		
Total	63	78705.750			

- ✓ SED 1.070
- ✓ LSD 2.152
- ✓ CV% 6.1