



Role of Hormonal Cross-talk and Signaling in the Postharvest Physiology of Lisianthus (*Eustoma Grandiflorum* Shinn.) Cut Flowers

A proposal submitted in fulfilment for the degree of
Doctor of Philosophy in Horticulture
In the University of Nairobi

Investigator:

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Introduction

- Horticulture is among the leading foreign exchange earners in Kenya.
- Horticulture overtook tea and even tourism to become the leading foreign exchange earner in the national economy of Kenya in the year 2007 (HCDA, 2007; Gray, 2008).
- However, the horticultural industry in Kenya is currently recovering from the twin adverse effects of the Icelandic volcanic ash cloud and extreme cold weather that affected all major international airports in Europe in 2010 and 2011 (HCDA 2010; Musembi, *et al.*, 2012).

Introduction cont'

- Kenyan horticultural subsector employs over 300,000 and 4.5 million people, directly and indirectly, respectively (Musembi, *et al.*, 2012).
- Lisianthus exports have dramatically increased in recent years, from a value of KES. 5.96 million in 1995, to a value of KES. 164.57 million in 2007 (Table I).
- Lisianthus cut flowers are among the top ten in popularity locally and internationally (Harbaugh, 2007; HCDA 2011).

Table I: Top ten cut flowers (by value) exported from Kenya in 1999 and 2007

| Crop Ranking 1999 | Value (Ksh.) | Crop Ranking 2007 | Value (Ksh.) |
|-------------------------------------|---------------------|----------------------------------|---------------------|
| 1. Roses | 5,519,864,824. | Roses | 21,556,017,152. |
| 2. Carnations (spray & standard) | 332,158,348. | Hypericum | 1,692,003,840 |
| 3. Statice | 251,705,574. | Carnations (spray & standard) | 958,041,664. |
| 4. <i>Alstroemeria</i> | 231,438,476. | Zantedeschia | 194,184,800. |
| 5. Lisianthus | 129,911,942. | Lisianthus | 164,568,144. |
| 6. Gypsophilla | 126,782,388. | <i>Alstroemeria</i> | 133,555,496. |
| 7. Chrysanthemum | 104,070,906. | Veronica | 129,230,336. |
| 8. Bupleurum | 53,543,755. | Arabicum | 124,124,652. |
| 9. Arabicum | 45,629,497. | Statice | 123,162,424. |
| 10. Veronica | 42,011,710. | Molucella | 72,963,280. |

(Source: HCDA statistics, 1999; 2007)

Introduction cont'

- The popularity of lisianthus has been attributed to the development largely in Japan, of excellent cultivars in a wide range of colours (Cho *et al.*, 2000; 2001; Reid, 2000; Harbaugh, 2007).
- However, for this new cut flower to continue expanding and maintaining its market share, the importance of research can not be overemphasized.

Introduction cont'

- Cross-talk in hormonal signaling pathways is an important topic in both cell and developmental biology in the 21st century (Eckardt, 2005).
- Various plant hormones affect overlapping processes and so, the expressed effect depends on specific hormone combinations rather than on the independent activities of each (Weiss and Ori, 2007).
- Before the year 1990, very little reference to cross-talk occurred in the literature in any area of biology (Eckardt, 2005).

Introduction cont'

- The term cross-talk defines points of regulatory interaction between two or more signal transduction pathways in living organisms (Eckardt, 2005).
- For the last two decades, various components of the signal transduction pathways of various plant hormones have been identified leading to the explanation of partial or entire signaling cascades (Weiss and Ori, 2007).
- Hormonal cross-talk acts at the hormonal response level as well as at the biosynthesis level, thus establishing a delicate response network (Weiss and Ori, 2007).

Introduction cont'

- Hormonal cross-talk in higher plants has been reported between all the major groups of hormones (Auxins, gibberellins, cytokinins, ethylene and abscisic acid) as well as between other hormones such as brassinosteroids and (Bouquin *et al.*, 2001) and jasmonate (Traw and Bergelson, 2003).
- The challenge now is to determine the mechanisms of cross-talk, or how all the signal transduction pathways interact and how they are co-ordinately regulated (Eckardt, 2005).
- It is clear, however, that the current state of knowledge is wanting considering the complex network of interactions between the various plant hormones (Weiss and Ori, 2007).

Introduction cont'

Problem Statement and Research Justification

- Ethylene is a gaseous hormone that triggers senescence and programmed cell death (Hurr *et al.*, 2010; Shahri and Tahir, 2011; Lombardi *et al.*, 2012).
- Lisianthus is ethylene sensitive (Ichimura *et al.*, 1998; Musembi, 2008). The current anti-ethylene pre-treatment silver-thiosulphate (STS) is a potent environmental pollutant under phase-out (Ichimura, 2005).
- The alternative 1-MCP is weak owing to its volatile nature (Serek and Sisler, 2001).

Introduction cont'

Problem Statement and Research Justification cont'

- Loss of leaf turgor, weak pedicels, premature leaf chlorosis, failure of majority of the florets to open, poor anthocyanin expression especially in newly opened florets, browning of stems due to biocide sensitivity, abscission failure of senescent florets and response to gravity, are some of the problems associated with the postharvest handling of lisianthus (Ichimura 1998; Ichimura *et al.*, 1998; Reid, 2000; Cho *et al.*, 2000; 2001; Musembi, 2008).

Introduction cont'

Problem Statement and Research Justification cont'

- Cut flower senescence is genetically programmed and mediated via hormonal (Chang, 2003) and developmental signaling therefore, vase life of cut flowers would probably be prolonged by reversing the detrimental effects of the senescence-signaling hormones (Musembi, 2008) and/or interrupting the developmental memory (Musembi *et al.*, 2012).
- Whereas ethylene and abscisic acid have been associated with aggravating the symptoms of senescence (Smart, 1994), cytokinin and gibberellin have been shown to delay senescence in various cut flowers (Mutui, 1998; Mutui *et al.*, 2001; Pinto *et al.*, 2007; Musembi, 2008).

Introduction cont'

Problem Statement and Research Justification cont'

- Musembi (2008) reported that, exogenous supply of cytokinin and/or gibberellin via xylem could improve the quality and vase life of lisianthus cut flowers beyond the ability of STS.
- Although, Musembi (2008) demonstrated that the response surface of the interaction between cytokinin and gibberellin was characterised by positive and negative interaction zones for various parameters, the modes of action remain unknown.

Introduction cont'

Problem Statement and Research Justification cont'

- Greenboim-Wainberg *et al.*, (2005) reported that cytokinin and gibberellin exclusively interact negatively and their cross-talk converges at SPINDLY/SPY proteins.
- However, the novel positive interaction between cytokinin and gibberellin in lisianthus cut shoots reported by Musembi (2008) and its molecular switching mechanism(s) are beyond the current state of knowledge, thus prompting this study.
- This study is expected to demonstrate the modes of action and thus enable predictable exploitation of this novel technology.

Introduction cont'

Objectives

- The main objective of this study is to establish the role of hormonal cross-talk and the mechanisms of hormonal signaling in the postharvest physiology of lisianthus cut flowers.
- The specific objectives are defined by the research questions listed below.

Introduction cont'

Research questions to guide this study

1. How does the exogenous application of combined cytokinin-gibberellin dosages influence the maintenance of cell membrane integrity and how does this relate to the water status of lisianthus cut flowers? How does this compare to reports on other plants?
2. How does the interaction between cytokinin and gibberellin influence the guard cells and how does this relate with water balance maintenance in lisianthus? How does this compare to reports on other plants?

Introduction cont'

Research questions to guide this study

3. How do endogenous hormone levels respond to the exogenous application of cytokinin/gibberellin solo or combined dosages in lisianthus compared to other plants?
4. What is the nature and implications of the response surface for BA/GA₃ hormonal combination treatments for BA and GA₃ on lisianthus vase life? How does this compare to reports in other plants?

Introduction cont'

Research questions to guide this study cont'

5. Does intra-shoot competition for water occur in harvested lisianthus and how does this compare to reports on other plants?
6. What are the modes of action and molecular switches involved in the water status regulation by cytokinin-gibberellin combined pre-treatment in lisianthus and how does this compare to reports on other plants?
7. Where do the interactions (positive and negative) between cytokinin and gibberellin converge in lisianthus compared to reports on other plants?

Materials and Methods

Site

- This study will be carried out in the Postharvest Physiology Laboratories, University of Nairobi, using plant materials obtained from a commercial grower. The laboratory will be lighted for a 12 hour photoperiod during the experimental duration, using Philips® 84 Watts cool white fluorescent tubes ($24 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD).

Materials and Methods cont'

Plant materials

- The cut lisianthus shoots (60 cm length) to be used in this study, will be grown under polyethylene green houses by the commercial grower and will be harvested at the mature bud stage (when one floret was ready to open).
- 'Flamenco Series' and 'Magic Series' lisianthus cultivars with single and double flower forms respectively, will be used in these experiments.

Materials and Methods cont'

Vase and Vial solutions

- Deionised water will be used to make preservative solutions. Each preservative solution will contain 0.7 mM NaOCl as germicide and 60 mM sucrose (Musembi, 2008), except for the deionised water control.
- The pH will be adjusted to 3.5, using citric acid (Musembi, 2008). Analytical grade (Sigma-Aldrich) BA (> 99%) and GA₃ (> 95%) will be used in this study.

Materials and Methods cont'

Treatments

- The hormonal treatments will be combined as shown in Table II.
- In addition the following will be used as controls: deionised water, STS, maleic hydrazide, abscisic acid (ABA), NAA and placebo.
- The zero hormone level (sugar, germicide and pH adjustment with citric acid as in all hormonal treatments) will be the placebo.

Materials and Methods cont'

Experimental design and data analysis

- This experiment will be set as a complete factorial: with the type of growth regulator as the fixed effect treatment; and the various dosages of the growth regulators as random effect treatments.
- Equally spaced random effect treatments will be chosen to enable analysis of the nature of response surface.
- The experiments will be laid out as Randomized Complete Block Design with 3 replicates. The blocking factor will be stem calliper.

Materials and Methods cont'

Experimental design and data analysis cont'

- Analysis of variance will be performed on the data, using the general linear model procedure of statistical analysis, using GENSTAT Statistical Package (Lane and Payne, 1996).
- Linear, quadratic and cubic orthogonal polynomials will be carried out and appropriate regression models used to examine the nature of the response to the various equally spaced random effect treatments such as GA₃ and BA (Steel and Torrie, 1980; Myers *et al.*, 2009).
- Multiple comparisons among means will be done using Tukey's Honestly Significant Difference (HSD) at 5% level of significance (Steel and Torrie, 1980).

TABLE II

Complete factorial treatment combinations of BA and GA₃ dosages

| | | BA Levels ((μ M)) | | | |
|-----------------------------------|----|------------------------|----|-----|-----|
| | | 0 | 70 | 140 | 210 |
| GA ₃ Levels (μ M) | 0 | × | × | × | × |
| | 5 | × | × | × | × |
| | 10 | × | × | × | × |
| | 15 | × | × | × | × |

Materials and Methods cont'

A) Response variates at the whole-shoot physiological level

Determination of vase life

- The vase life of the lisianthus cut flowers will be determined according to Musembi, (2008).

Determination of number of days to loss of pedicel turgor

- Number of days to loss of pedicel turgor will be recorded according to Musembi (2008).

Chlorophyll content determination

- The chlorophyll content will be determined according to Musembi (2008).

Water balance

- Water balance = Water uptake – Transpiration. Water balance and its components will be determined according to Musembi (2008).

Materials and Methods cont'

A) Response variates at the whole-shoot physiological level cont'

Internal Water relations

- Evaluation of intra-shoot competition for water between inflorescences and leaves will be carried out according to He *et al.*, (2006).

Determination of carbon dioxide production rate

- The rate of carbon dioxide production will be determined according to Musembi (2008).

Determination of ethylene production rate

- The rate of ethylene production will be determined according to Musembi (2008).

Materials and Methods cont'

B) Responses at the cellular level

Determination of maintenance of cell membrane integrity

- The maintenance of the integrity of the cell membrane will be evaluated according to Singh (2008); in parallel with the whole-shoot physiology study.

Determination of stomatal aperture

- The stomata aperture will be determined using the abaxial epidermal peels from treated shoot leaves examined under light microscopy with mounted digital camera according to Tanaka *et al.* (2005); in parallel with the whole-shoot physiology study.

Materials and Methods cont'

C) Responses at the molecular level

Responses at the molecular level shall be investigated in parallel with the whole-shoot physiology study. An overview of differential up-regulation and down-regulations of genes following the solo or combined cytokinin-gibberellin pre-treatment of lisianthus shoots will be carried out using mRNA or protein Microarray Scanner e.g. GeneAtlas[®] using Partek Express, Affymetrix Edition[®] for whole-genome expression or other appropriate gene-expression database and software.

Materials and Methods cont'

C) Responses at the molecular level cont'

Quantification of mRNAs and Proteins involved in hormonal signal transduction pathways and hormonal cross-talk

- mRNA Microarray analysis will be followed by differential expression of mRNAs of the genes of interest, quantified using qRT-PCR e.g. ABI-7700[®] instrument or other appropriate qRT-PCR equipment.
- Explant samples obtained from treated shoots when phenotypes of parallel whole-shoot physiology study indicate occurrence of hormonal interaction.
- Total RNA will be extracted from 1 g aliquot of frozen petal tissue using Invitrogen (Carlsbad, CA, USA) following the manufacturer's instructions.

Materials and Methods cont'

C) Responses at the molecular level cont'

Quantification of mRNAs and Proteins involved in hormonal signal transduction pathways and hormonal cross-talk

- qRT-PCR will be used to monitor differential gene expression in the loci of interest, which include: rate-limiting hormonal biosynthesis genes; *ISOPENTENYL TRANSFERASE* loci, *GA1-3* homologs, *ACC SYNTHASE* loci, *9-CIS-EPOXYCAROTENOID DIHYDROGENASE* loci, and *FLAVIN MONOOXYGENASE-LIKE ENZYME (YUCCA)* loci, for cytokinin, gibberellin, ethylene, ABA and auxin, respectively.
- Expression of key hormonal signal mediating protein mRNAs, will also be quantified using qRT-PCR.

Materials and Methods cont'

C) Responses at the molecular level cont'

Quantification of signal mediating Proteins involved in hormonal cross-talk

- Protein Microarray analysis will be followed by measurement of the changes in activity of hormonal signal mediating proteins: DELLA family, SPY/SPINDLY and KNOX groups using real-time immunoassay e.g. DotLab[®] instrument or other appropriate quantitative immunoassay techniques.
- Micro-RNA (miRNA) involvement in hormonal cross-talk will be investigated using Microarray instrument.

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Expected Outputs and Outcomes

Outputs

- PhD thesis
- Published original research papers

Outcomes

- Novel technologies and preproduction prototypes (patents)
- Qualified agricultural scientist(s)
- Positive contribution towards Kenya Vision 2030

YEARLY BUDGET BREAKDOWN

| YEAR: 1 | | | | | | |
|---|----------|----------|-----------|------------------|--|--|
| Item Description | Quantity | Units | Unit Cost | Cost per Batch | | |
| Special HPLC columns | 3 | sets | 50,000 | 150,000 | | |
| Special glass vases | 60 | pcs | 500 | 30,000 | | |
| Plant growth regulators | 8 | g | 10,000 | 80,000 | | |
| Hormonal-action inhibitors | 5 | g | 8,000 | 40,000 | | |
| Plant materials, reagents, DIW (lot 1) | 1 | lot | 40,000 | 40,000 | | |
| Sourcing lisianthus cutflowers (trip 1) | 1 | trip | 30,000 | 30,000 | | |
| Postharvest physiology extra personnel | 50 | man-days | 500 | 25,000 | | |
| Printing, | 210 | pgs | 10 | 2,100 | | |
| Photocopying | 1000 | pgs | 2 | 2,000 | | |
| Binding | 3 | sets | 300 | 900 | | |
| Novel microscope embodiment | 1 | sets | 250,000 | 250,000 | | |
| Subtotal | | | | 650,000 | | |
| YEAR: 2 | | | | | | |
| Item Description | Quantity | Units | Unit Cost | Cost per Batch | | |
| Scanning electron microscope kits | 2 | sets | 20,000 | 40,000 | | |
| Special gas analyser kits | 2 | sets | 25,000 | 50,000 | | |
| Scarpels, gloves, masks, M. slides | 1 | lot | 10000 | 10,000 | | |
| Sourcing lisianthus (trip 2) | 1 | trip | 10,000 | 10,000 | | |
| Plant materials, reagents, DIW (lot 2) | 1 | lot | 10,000 | 10,000 | | |
| Postharvest physiology extra personnel | 30 | man-days | 500 | 15,000 | | |
| RNA extraction kits (lot 1) | 1 | sets | 70,000 | 70,000 | | |
| qRT-PCR reaction kits (lot 1) | 2 | sets | 80,000 | 160,000 | | |
| Printing, | 1210 | pgs | 10 | 12,100 | | |
| Photocopying | 1000 | pgs | 2 | 2,000 | | |
| Binding | 3 | sets | 300 | 900 | | |
| microarray/qRT-PCR extra personel | 40 | man-days | 500 | 20,000 | | |
| Subtotal | | | | 400,000 | | |
| YEAR: 3 | | | | | | |
| Item Description | Quantity | Units | Unit Cost | Cost per Batch | | |
| RNA extraction kits (lot 2) | 1 | sets | 70,000 | 70,000 | | |
| qRT-PCR reaction kits (lot 2) | 3 | sets | 80,000 | 240,000 | | |
| Printing, | 2980 | pgs | 10 | 29,800 | | |
| Photocopying | 3000 | pgs | 2 | 6,000 | | |
| Binding | 14 | sets | 300 | 4,200 | | |
| microarray/qRT-PCR extra personel | 60 | man-days | 500 | 30,000 | | |
| Sourcing lisianthus (trip 3) | 1 | trip | 10,000 | 10,000 | | |
| Plant materials, reagents, DIW (lot 3) | 1 | lot | 10,000 | 10,000 | | |
| Subtotal | | | | 400,000 | | |
| Grand Total | | | | 1,450,000 | | |

Work Plan

Nicodemus Ndawa Musembi (DAAD Code No. A/10/02840)

PhD in Horticulture Student, University of Nairobi

Work Plan and Time Table

| Month/Year | Sep-09 | Oct-09 | Nov-09 | Dec-09 | Jan-10 | Feb-10 | Mar-10 | Apr-10 | May-10 | Jun-10 | Jul-10 | Aug-10 |
|------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
|------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|

| | | | | | | | | | | | | |
|------------|--|-----|-----|------------------|-----|-----|-------------------|-----|-----|---------------------------------|----|----|
| Activities | PLR | PLR | PLR | PLR | PLR | PLR | PLR | PLR | PLR | PLR | DP | DP |
| | Preliminary research | | | First Experiment | | | Second experiment | | | Data analysis Research paper | | |
| | PLR = Physiological-level hormonal cross-talk research | | | | | | | | | | | |

| | | | | | | | | | | | | |
|-----------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Comment: | Done | Done | Done | Done | Done | Done | Done | Done | Done | Done | Done | Done |
|-----------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|

| Month/Year | Sep-10 | Oct-10 | Nov-10 | Dec-10 | Jan-11 | Feb-11 | Mar-11 | Apr-11 | May-11 | Jun-11 | Jul-11 | Aug-11 |
|------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
|------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|

| | | | | | | | | | | | | |
|------------|---|-----|-----|------------------|-----|-----|-------------------|-----|----|---------------------------------|----|----|
| Activities | CLR | CLR | CLR | CLR | CLR | CLR | CLR | CLR | DP | DP | DP | DP |
| | Preliminary research | | | First Experiment | | | Second experiment | | | Data analysis Research paper | | |
| | CLR = Cellular-level hormonal cross-talk research | | | | | | | | | | | |

| | | | | | | | | | | | | |
|-----------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Comment: | Done | Done | Done | Done | Done | Done | Done | Done | Done | Done | Done | Done |
|-----------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|

| Month/Year | Sep-11 | Oct-11 | Nov-11 | Dec-11 | Jan-12 | Feb-12 | Mar-12 | Apr-12 | May-12 | Jun-12 | Jul-12 | Aug-12 |
|------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
|------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|

| | | | | | | | | | | | | |
|------------|--------------|-----|----------|----------|------|------|----------------|------|-------------------------------|------|----------------|------------|
| Activities | MLR | MLR | MLR | MLR | FMLR | FMLR | FMLR | FMLR | FMLR | FMLR | DF | |
| | Prel Resear. | | 1st Expt | 2nd Expt | | | | | | | Thesis Defense | |
| | | | | DP | DP | DP | Research paper | | Thesis writing and submission | | | Graduation |

| | | | | | | | | | | | | |
|-----------------|--|--|--|--|--|--|--|--|--|--|--|--|
| Comment: | | | | | | | | | | | | |
|-----------------|--|--|--|--|--|--|--|--|--|--|--|--|

MLR = Molecular-level hormonal cross-talk research (**UoN - Kenya**)

FMLR = Further Molecular-level hormonal cross-talk research (**Leibz University of Hannover - Germany**)