



**UNIVERSITA' DEGLI STUDI DI
CATANIA**

Dipartimento di Scienze Biologiche, Geologiche e Ambientali

**DOTTORATO DI RICERCA IN
BIOLOGIA EVOLUZIONISTICA E DELL'AMBIENTE**

Coordinatore: Prof. Salvatore Saccone

GIUSEPPE DIEGO PUGLIA

**Meccanismi fisiologico-molecolari di
germinazione in specie spontanee**

Tesi di dottorato

Tutor: Prof. Pietro Pavone

Co-Tutor: Dr. Peter Toorop

XXVI ciclo

AA.AA. 2010÷2013



**UNIVERSITA' DEGLI STUDI DI
CATANIA**

Dipartimento di Scienze Biologiche, Geologiche e Ambientali

**DOTTORATO DI RICERCA IN
BIOLOGIA EVOLUZIONISTICA E DELL'AMBIENTE**

Coordinatore: Prof. Salvatore Saccone

GIUSEPPE DIEGO PUGLIA

**Physiological and molecular
mechanisms of seed germination in
weed species**

Ph.D. thesis

Tutor: Prof. Pietro Pavone

Co-Tutor: Dr. Peter Toorop

XXVI cycle

AA.AA. 2010÷2013

Abstract

Seed dormancy provides a mechanism for plants to delay germination until conditions are optimal for survival of the next generation. Knowledge of dormancy release and dormancy induction requirements is important to increase our understanding about the entire process. Furthermore, assess the association between gene expression patterns and physiological phase in dormancy is a major goal to allow a positive definition of this condition.

In *Chrysanthemum coronarium* var. *concolor* and var. *discolor*, anatomical and germination analyses showed that non-deep physiological dormancy (PD) is the more plausible type of dormancy for both of them. The genetic composition of *Chrysanthemum coronarium* varieties was characterized using Inter Simple Sequence Repeat (ISSR) PCR together with a DNA-Barcoding approach.

Seed priming treatments were used in a non-dormant species, *Leucanthemum vulgare*, to induct secondary dormancy at specific temperature range and a long-priming treatment was used for its release. Fluridone and giberellic acid were tested to investigate the metabolic pathway involved in the onset of secondary dormancy and in its maintenance.

Five probable housekeeping genes, useful as reference genes for quantitative PCR (qPCR) analysis in seed tissue, were identified in *Chrysanthemum coronarium* and in *Leucanthemum vulgare*, isolated and confirmed with comparative analysis with homologous genes in model species. One radicle-protrusion associated gene, *EXP* (*Expansin*), was identified in the two species, isolated and confirmed. Three dormancy-associated genes, *DOG1* (*Delay Of Germination 1*), *FLC* (*Flowering Locus C*), *HUB2* (*Histone Mono-Ubiquitination 2*) were identified in *Chrysanthemum coronarium* and in *Leucanthemum vulgare*.

Table of contents

Introduction.....	7
Seed germination.....	7
Radicle growth.....	8
Seed dormancy.....	8
Physical dormancy (PY).....	9
Physiological dormancy (PD).....	9
PD deep	10
PD intermediate.....	10
PD non-deep.....	10
Endosperm-weakening and embryo growth potential.....	11
Molecular aspects of seed dormancy.....	12
Dormancy release.....	13
Seed priming.....	14
Hydro-priming	14
Osmo-priming	14
<i>Chrysanthemum coronarium</i>	16
Species description and distribution	16
Heterocarpy and dormancy in Asteraceae.....	17
Seed germination.....	18
<i>Leucanthemum vulgare</i>.....	19
Species description and distribution.....	19
Seed germination.....	21
Varietal genotyping.....	23
Aim of the thesis.....	25
Materials and Methods.....	26
Study sampling.....	26
Germination experiments.....	27
Seed excision.....	28
Water uptake.....	28
Anatomical analyses of pericarp tissue.....	28
Germination in dark.....	28
Cold stratification.....	29
Dry storage.....	29
Dry after ripening.....	29
Wet warm stratification.....	29
Seed osmo-priming	29
Seed hydro-priming.....	30
ABA synthesis.....	30
Incubation in agitated water.....	30
Giberellic acid.....	30
Statistical analyses.....	31
Molecular analyses.....	32
<i>Chrysanthemum coronarium</i> varietal genotyping.....	32
DNA extraction.....	32
ISSR profiling.....	32
rbc-L gene amplification and sequencing.....	33
mat-K gene amplification and sequencing.....	34
Data analysis.....	34
Identification and isolation of reference and dormancy associated genes in <i>C. coronarium</i> and <i>L. vulgare</i>	35
Reference genes.....	35
Dormancy associated genes	35
DNA extraction.....	36
RNA extraction.....	36
cDNA first strand synthesis.....	37
PCR products cloning	37
Sequences analysis.....	38

Results.....	39
Physiology of germination of <i>Chrysanthemum coronarium</i>	39
Germination behaviour	39
Seed excision.....	40
Comparison of the germination behaviour of the two varieties.....	41
Water uptake	42
Anatomical analyses of pericarp tissue.....	42
Germination in dark.....	45
Cold stratification.....	46
Dry storage.....	48
Dry after ripening.....	49
Wet warm stratification.....	51
ABA synthesis.....	56
Genotyping of <i>Chrysanthemum coronarium</i> <i>concolor</i> and <i>discolor</i> varieties.....	57
DNA extraction	57
ISSR analyses.....	59
Sequencing of <i>rbcl</i> and <i>mat-k</i> regions.....	60
Physiology of germination in <i>Leucanthemum vulgare</i>.....	63
Germination behaviour	63
Seed osmo-priming	63
Hydro-priming.....	65
ABA synthesis.....	66
Isolations of dormancy genetic markers in <i>Leucanthemum vulgare</i> and <i>Chrysanthemum coronarium</i>.....	69
Genes selection.....	69
DNA extraction.....	71
RNA extraction	71
PCR products cloning with TA-Vector.....	72
Sequences analyses.....	77
Phosphoglycerate kinase (PGK) gene.....	77
18s gene:.....	78
UBC 21 gene.....	79
UBC 8-28 gene.....	80
UBC 9-10 gene.....	81
UBC sequences:.....	82
Expansin gene:.....	83
Discussion.....	84
<i>Chrysanthemum coronarium</i> germination physiology.....	84
<i>Chrysanthemum coronarium</i> varietal genotyping.....	85
<i>Leucanthemum vulgare</i> germination physiology.....	86
Cloning of reference and dormancy associated genes:.....	87
Conclusions.....	88
Reference cited.....	89

Introduction

Seed traits are recognized as decisive adaptations of spermatophytes that are subjected to natural selection and optimize plant fitness (Fenner 2005), (Donohue 2010). Among these traits, seed dormancy is considered one of the main factors determining the adaptive value of germination (W. E.-M. Finch-Savage 2006) (Venable 2007) (Donohue 2010). Upon dispersal, seeds may show total (i.e. no germination under any conditions) or conditional dormancy (i.e. germination under a restricted set of conditions) (J. M. Baskin 2004). In response to certain environmental signals (the so-called dormancy breaking factors), seeds gradually lose dormancy and their range of germination conditions broadens until eventually they become non-dormant. This stimulus can be related to the season of major risk for the seedling in many species (Vleeshouwers 1995) (Mattana 2012) (Wagmann 2012).

Seed germination

Seed germination process starts with water imbibition of a dry seed and ends with radicle protrusion (W. E.-M. Finch-Savage 2006) (Bassel 2011). The elongation of embryonic axis protruding through the endosperm tissue is clear evidence of the completion of germination. From the physiological point of view, germination consists of reactivation of metabolism, followed by protein synthesis and radicle cells extension (J. D. Bewley 1997). Metabolism resumption is triggered by water entrance in seed tissues, which activates a sequence of enzymatic mechanisms.

Water imbibition is a tri-phasic process. The phase I is characterized by a rapid water uptake, with a consequent immediate leakage of solutes (Crowe 1992), followed by mitochondria repairing and a resumption of principal metabolic pathways (Botha 1992) (J. D. Bewley 1994). Within 3 – 6 hours after imbibition the mRNAs transcribed during seed development are degraded and a distinct germination transcriptional program commences (K. O. Nakabayashi 2005). Conversely, at the same time, the amount of new transcripts increases gradually to support cell metabolism for seed germination (K. O. Nakabayashi 2005) (Carrera 2007). In the phase II water absorption is prevented, DNA is repaired to correct the reduction in DNA integrity resulting from storage in the dry state (Osborne 1994), and DNA replicative synthesis takes place (Zlatanova 1987).

In the phase III the increase in osmotic concentration promoted by the hydrolysis of storage solutes lead to a further water uptake. At the end of this phase radicle extension, through the structures surrounding the embryo, occurs and this is the event that terminates germination and marks the commencement of seedling growth. This extension may or may not be accompanied by cell division, but it always requires the yielding of hypocotyl cell walls (Cosgrove 1997) (J. D. Bewley 1997).

Radicle growth

Radicle growth may be exerted by a decrease in water potential of the seed (ψ_{π}), cell wall-loosening of tissues surrounding the radicle tip or by a partial wall degradation allowing elongation of the embryonic axis driven by turgor ψ_p (J. D. Bewley 1997) (Koornneef 2002). Previous authors (Pritchard 1993) (Wu 1994) observed an increase of activity of xyloglucan endotransglycosylase activity (XET) (which is decreased in presence of Fluridone and enhanced by ABA) specifically in the elongation zone with a low ψ_w . Moreover, a class of proteins named *expansins* was shown (Cosgrove 1997) (McQueen-Mason S. 1993) to induce cell wall extension in concert with a variety of enzymes that cut and restructure the wall in cucumber (McQueen-Mason 1995) (Cosgrove 1997) and in rice cultivars (Magneschi 2009). In tobacco, endosperm β -Glucanases induction was observed to be positively correlated with endosperm and testa rupture (G. F.-L. Leubner-Metzger 1995). This hypothesis was supported with the application of hormones that inhibit (ABA) or promotes (GA) seed germination (G. F.-L. Leubner-Metzger 1995): ABA presence prevents radicle cell wall extension, whilst it does not interfere with ψ_s , solute potential and with water uptake.

Seed coats and surrounding structures may have a further influence on the ability of a seed to germinate interfering with water uptake, gas exchange, diffusion of endogenous inhibitors, or by mechanical restriction of embryo growth (Clark 1989) (Durán 1989) (C. C. Baskin 1998). Conversely, even the endosperm tissue may restrict embryo growth: in pepper mechanical resistance of the endosperm appeared to affect germination (Watkins 1983).

Seed dormancy

Seed dormancy, could be defined as a block to the completion of germination of an intact viable seed under favourable conditions (W. E.-M. Finch-Savage 2006). It forms a

protection mechanism for species survival, avoiding undesirable germination during unfavourable environmental conditions for seedling growth (A. G.-M. Linkies 2010).

Recent seed physiology literature (J. M. Baskin 1998) (J. M. Baskin 2004) discriminates five classes of dormancy depending on which factor causes seed dormancy: physical (PY), physiological (PD), morphological (MD), morphophysiological (MPD) and combinational (PY+PD) dormancy. In this manuscript, only physical and physiological dormancy will be introduced because of their pertinence with the species presented in this study.

Physical dormancy (PY)

In physical dormancy seed/fruit covering layers impose dormancy preventing embryo/seed imbibition (J. M. Baskin 2000). Water-impermeable seed (or fruit) is obtained during maturation drying of the seeds (Van Staden 1989) (C. C. Baskin 1998) by a layer of palisade cells acting as a physical barrier against water entrance. Heavy lignified cells, waxes or suberin-cutin usually make up this cell barrier.

In PY once the seed or fruit becomes permeable to water by a loss of barrier integrity, the seed can imbibe and germinate over a wide range of temperatures (C. C. Baskin 1998) (J. M. Baskin 2000). As a consequence, once the physical barrier has been disaggregated, it generally cannot revert to impermeability (Hamley 1932). Release of physical dormancy is usually exerted by external factors that could affect impermeable cell layer integrity. In nature, these factors include high temperatures, widely fluctuating temperatures, fire, drying, freezing/thawing and passage through the digestive tracts of animals (C. C. Baskin 1998).

Physiological dormancy (PD)

In physiological dormancy (PD) a block of germination occurs, and it is removed only with the presence of specific environmental cues (W. E.-M. Finch-Savage, Seed dormancy and the control of germination 2006). Within *A. thaliana* physiological dormant seed the ABA- and GA-biosynthesis and catabolism were modelled (C. T.-S. Cadman, Gene expression profiles of Arabidopsis Cvi seeds during dormancy cycling indicate a common underlying dormancy control mechanism 2006) (W. E. Finch-Savage 2007), where hormones catabolism and metabolism are influenced by prevailing environmental conditions. This balance can make the difference between germination and the seed remain dormant.

Furthermore, presence of inhibitors in seed coat layers or endosperm-weakening can inhibit embryo growth.

Depending on the intensity of physiological dormancy in seeds, three different types of PD were identified.

PD deep

PD-deep seeds excised embryos do not grow or will produce abnormal seedlings. They usually need long pre-chilling treatments and chemicals do not exert any remarkable effect on seed germination. *Pyrus* seeds re-enter in dormancy during post-stratification drying after a pre-chilling treatment (Shen 2001).

PD intermediate

Embryos excised from PD-intermediate seeds produce normal seedlings. Usually a long pre-chilling, up to 6 months, is necessary. GA, kinetin or thiourea could reduce cold stratification period.

PD non-deep

Non-deep physiological dormancy is the most common physiological dormancy class (J. M. Baskin 2004). Excised embryos produce normal seedling, and dormancy is usually released after a short cold stratification time period. Moreover, these seeds, depending on their distribution area, they need a cold or warm stratification to release dormancy. This, could be broken by chemicals (potassium nitrate, thiourea, kinetin, ethylene, and gibberellins. Non-deep physiological dormant seeds, usually, need light to germinate.

Seeds with a PD non-deep gradually lose dormancy and enter in a conditional dormancy state (Naylor 2008). At the beginning of this stage, an increasing amount of seeds is able to germinate over a narrow range of temperatures. As the conditional dormancy is gradually lost an increasing proportion of seeds germinate over a more ample range of environmental conditions (J. M. Baskin 2004).

There is a general agreement on the identification of five types of non-deep PD proposed by Baskin & Baskin (J. M. Baskin 2004), which is based on the patterns of change in physiological responses to temperature during dormancy break (see Figure 1). In the first type temperature range at which seeds can germinate gradually increases from low to high temperatures along with dormancy release. *Arabidopsis thaliana* is a species with non-deep PD type 1 (Meinke 1998).

In the second type, during dormancy release temperature range required for germination decreases as a continuum from high to low, example: *Helianthus annuus* (Corbineau 1990) (M. T. Le Page-Degivry 1990).

In type 3 temperature range for germination increases during dormancy release as a continuum from medium to high and low, example: *Aster ptarmacoides* (M. B. McDonald 2005).

In non-deep PD type 4 and 5 temperature and dormancy pattern change does not result in continuum trend, but non-dormant seeds germinate only at high temperature (type 4), example: *Callicarpa americana* (M. B. McDonald 2005) or low temperature (type 5), example: *Gentianella quinquefolia* (J. M. Baskin 2004).

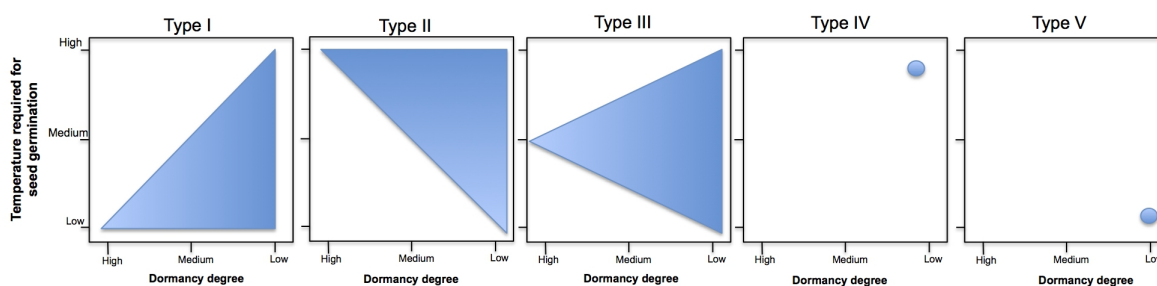


Figure 1 Five types of non-deep physiological seed dormancy (PD) according to Baskin and Baskin (2004).

Endosperm-weakening and embryo growth potential

Differently from physical dormancy imposed by a physical barrier to water entrance, a mechanical constraint exerted by endosperm combined with low embryo growth potential can result in seed dormancy. This condition falls in non-deep physiological dormancy and it can be released by light and GA, which increase embryo osmotic potential and/or reduce endosperm weakening (Casal 1998), (G. M. Leubner-Metzger 2001) (Sanchez 2004) (Kucera 2005). The force required to break seed coats depends on the different seed surrounding layers features and it can range from 9.9MPa in *Pancrethium maritimum* to 133.2MPa in *Iris lorteti* (Blumenthal 1986). Appropriate germination or dormancy break conditions as presence of light (Scheibe 1965) or darkness (S. Chen 1968), giberellic acid (J. M. Baskin 1971), incubation temperatures (Juntilla 1973) or cold stratification (Carpita 1983) could promote embryo growth potential enough for radicle protrusion through the seed coat (C. C. Baskin 1998). Moreover, seed coat scarification may increase germination percentage removing the mechanical constraint as in cucumber and in sunflower seeds, where, after scarification, a 50% increase of

germination was observed in light at an increased osmotic potential (McDonough 1967). In tomato the endosperm cap weakening is a biphasic process correlated with endo- β -mannase activity for the first phase and controlled by ABA in the latter one (Toorop 2000).

Molecular aspects of seed dormancy

Notwithstanding the function of dormancy in different plant species to spread germination across time avoiding unfavourable environmental condition for seedling growth (C. C. Baskin 1998) there is a common underlying molecular mechanism that manage seed dormancy in plants (C. S.-S. Cadman 2006) (A. G.-M. Linkies 2010). However, the regulation of this process at the molecular level is far to be unveiled (Footitt 2011). Recent studies on non-deep physiological dormancy in *A. thaliana* have provided new insights (C. T.-S. Cadman, Gene expression profiles of Arabidopsis Cvi seeds during dormancy cycling indicate a common underlying dormancy control mechanism 2006) (W. E. Finch-Savage 2007) (S. D.-S.-S. Footitt 2011) showing that ABA and GA hormones balance is finely tuned at molecular level. As an example, it has been shown that the expression of genes involved in the ABA/GA level regulation is sensitive to seed maturation temperature (S. L. Kendall 2011). Genetic approaches have led to the identification of specific loci correlated with altered dormancy, and -omics approaches in many species have identified numerous transcripts and proteins correlated with dormant versus non-dormant seeds (W. E.-M. Finch-Savage, Seed dormancy and the control of germination 2006) (L. J. Bentsink 2006) (G. C. Chiang 2009) (Bassel 2011). *DELAY OF GERMINATION 1 (DOG1)* gene is a major quantitative trait gene more specifically involved in seed dormancy and germination timing (L. J. Bentsink 2006), it is so far only known within the Brassicaceae and its expression appeared to be the dominant factor influencing ABA signalling (S. H.-S. Footitt 2013) (Graeber 2013). Other studies (G. C. Chiang 2011) (S. C.-S. Footitt 2014) have shown *DOG1* expression to be negatively correlated to soil temperature which protein accumulation drives changes in germination potential acting as a timer for seed dormancy release (K. B. Nakabayashi 2012). Furthermore, the expression of *GA3ox1* gene (GA biosynthesis) was observed to follow seed water potential cycle decreasing upon burying, with a subsequent continuum increase until dormancy release (S. H.-S. Footitt 2013). Other researches shown the gene *FLC* (*Flowering Locus C*) to have a role in germination timing with a similar expression pattern as *GA3ox1* gene (G. C. Chiang 2009) (Chiang G. C. 2013).

Analysis on *Arabidopsis thaliana* mutants with reduced dormancy (rdo) (Peeters 2002) and successive investigations (Liu 2007) showed that *HISTONE MONOUBIQUITINATION* (*HUB1*) protein influences gene expression of dormancy-related genes. Recently in *Castanea sativa* a clear association of genes encoding histone mono-ubiquitinase *HUB2* with dormancy behaviour was shown (Santamaría 2011).

Dormancy release

Recent speculations on seed dormancy release mechanisms have led to consider dormancy as a continuum process, where the expression of dormancy-related genes is highly sensitive to the soil temperature history, nitrate, light and GA (C. S.-S. Cadman 2006) (W. E.-M. Finch-Savage, Seed dormancy and the control of germination 2006) (W. E. Finch-Savage 2007) (S. D.-S.-S. Footitt 2011) (W. E. Finch-Savage 2012). This continuum is made up by layers of dormancy being progressively removed by environmental cues until only light is required, in the absence of which seeds remain dormant and enter into another dormancy cycle as the seasons change (W. E. Finch-Savage 2012) (S. C.-S. Footitt 2014). Therefore, any cue that enlarges the environmental required conditions allowing germination has to be intended as a release factor (W. E. Finch-Savage 2007).

Dormancy-release factors are usually related to the specific environment where the species is spread. So that species adapted to regions with a dry season where lack of water represents a serious obstacle for seedling growth, the dry after-ripening, i.e. a period of dry storage for usually several months (J. D. Bewley 1997) (Kucera 2005) (Probert 2000), is often the major dormancy release factor.

During dry storage physiological changes takes place within the seed, which result in a decrease in the level of dormancy and, as consequence, germination requirements become less specific (Probert 2000). In particular, in tobacco dormant seeds, dry after-ripening treatment led to a widening of the temperature range for germination, a decrease in ABA level within the seed and an increase in GA sensitivity, a loss of the requirement for nitrate, and an increase of germination promptness (G. Leubner-Metzger 2005).

Furthermore, even cold and warm incubation of pre-imbibed seeds can play a role in both release and induce dormancy (Probert 2000). In winter annuals high winter temperatures release dormancy and low summer temperatures induce dormancy (J. M. Baskin 1978) (J. M. Baskin 1978). On the contrary, in summer annuals low winter temperatures release dormancy, whereas high summer temperatures induce dormancy (J. M. Baskin 1977) (J. M. Baskin 1987).

Seed priming

Among treatments that could alter seed physiology, which, in turn, could promote dormancy release, seed priming is widely used in crops as a standard technique (S. G. Kaur 2005) (Guan 2009). It was shown to widen the temperature range suitable for germination (McDonald 2000) (Jisha 2013), and provide faster and synchronous seedling emergence (Taylor 1990) accomplish uniform emergence, and give better crop stand in many horticultural and field crops (Ashraf 2005).

It consists in the acquisition of the potential to rapidly imbibe and revive the seed metabolism thus enhancing the germination rate (McDonald 2000).

Seed priming could be implemented with different priming factors: water (hydropriming), osmoticum (osmopriming), chemicals (chemical priming), etc. In this manuscript only hydropriming and osmopriming will be introduced because of their pertinence with the presented experimental procedure.

Hydro-priming

Hydro-primed seeds are immersed in water and kept at appropriate temperature for a determined length of time (Kaya 2006). Afterwards seeds are dried to their original weight (Bennett 1987) (Thomas 2000). Hydro-priming promotes metabolism reactivation even if germination is prevented (S. M. Basra 2003). Protoplasm of hydro-primed seeds is found to have a lower viscosity and exhibit higher permeability to water and nutrients and also hold water against dehydrating forces (Thomas 2000). In onion germination hydro-priming was found to be the most effective method for improving seed germination (Caseiro 2004). In *Zea mays* L. priming widened germination temperature range and shortened germination time without decreasing final percentage emergence (W. E. Finch-Savage 2004).

Osmo-priming

Osmo-primed seeds are exposed to low-water-potential solutions to restrict the rate and extent of imbibition. This process prolongs the early imbibition phase promoting a gradual progression of various pre-germinative metabolic activities (Bradford 1986).

Polyethylene glycol (PEG) is the most common agent used to create low-water potential solution because of its nontoxic nature and large molecular size, which lowers water potential without penetrating into the seeds during soaking (Thomas 2000). In comparison with hydropriming, osmopriming can preserve plasma membrane structures resulting in

better germination frequency because of controlled long hydration in seeds (Jett 1996). In rice, osmopriming treatment was observed to enhance starch hydrolysis triggering a series of events leading to more vigorous seedling production, improved allometric, kernel yield and quality attributes (Farooq 2006).

In contrast to the copious literature on positive effects of seed priming, to date just few studies report a negatively interference of this treatment with germination in non-dormant seeds. In Amitiphale *et al.* (D. S. Amritphale 2000) cucumber seeds imbibition within an osmoticum allowed examining the dormancy breaking in isolation from the germination process. In fact the osmotic treatment caused germination inhibition, whereas a seed exposure to far red light induced secondary dormancy. Researchers hypothesized that change in plasma membranes fluidity led to the transition from dormant to germinable state.

Chrysanthemum coronarium

Species description and distribution

Chrysanthemum coronarium L. (family Asteraceae; tribe Anthemideae; genus *Chrysanthemum*) is an aromatic heterocarpic winter annual 20-60 cm high with a straight, hairless and branched trunk; leaves are light green, and deeply lobate. The chorological type is Steno-Mediterranean, see Figure 2 (Pignatti 1982) (Meusel 1965).

It is usually found as weed in diverse crops, along roadsides, on field margins and on abandoned fields (Figure 3). Although essentially Mediterranean this species is nowadays naturalized far beyond their ‘native’ range and since its abundance and persistence it was reported as invasive plant species (Cook 2011) (Schaefer 2011). In Sicily, its phytosociological association was described by earlier authors (Ferro 1980) (Brullo 1985) (Brullo 2001) (Ferro 2004).

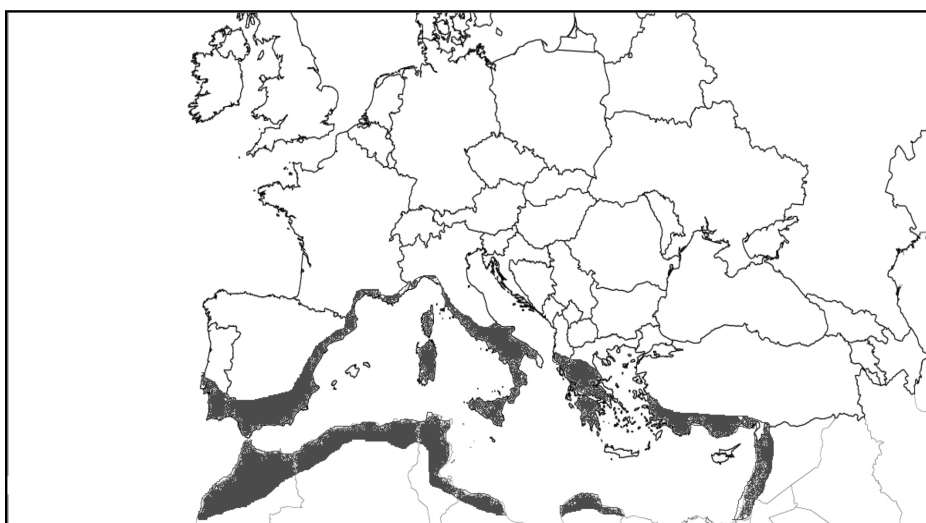


Figure 2 *Chrysanthemum coronarium* distribution according to H. Meusel. Figure re-drawn from (Meusel 1965).

Dumont d’Urville described two varieties under *Chrysanthemum coronarium*: var. *concolor* d’Urv., from the island of Malta, with yellow ray florets (“*radio discoque luteis sola reperitur inter segetes insulae Melitae*”), and var. *discolor* d’Urv., from the Aegean islands, with white ray florets (“*radio albo discoque luteo copiosè occurrit in insulis Archipelagi*”) (d’Urville 1822).

The two entities appear to be widespread in the Mediterranean region and show no obvious correlation with geographic distribution (Turland 2004). Recently, investigation on the

different population occurrence of the two varieties shown that the *discolor* variety occurs in thermo- and meso-Mediterranean areas of Greece, Italy, Spain, Portugal and the north of Morocco, whereas no occurrence of the *concolor* var. was reported in the meso-Mediterranean environment (E. C.-O. Cano 2012) (Cano-Ortiz 2007). In the thermo-Mediterranean belt the populations of both variants tend to appear separately with only occasional concurrences (E. C. Cano 2013).

Furthermore, a kariomorphological study (El-Twab 2008) on *Chrysanthemum coronarium* varieties shown that, although the two varieties are both $2n = 18$, as previously reported for *Chrysanthemum coronarium* (Bhattacharyya 1977) (Pavone 1981) (Razaq Z.A. 1988) (Nirmala A. 1986) (Vogt R. 1993) (Kaul M.L. 1995) (Strother 1997) (Carr 1999), they differ in the arrangement of two chromosomes (submedian-centromeric in *discolor* and median-centromeric in *concolor*).

In other studies, basing on the disposition of the intercostal glands, the colour of the ligules, and the size and wings of the cypselas of the flosculous flowers and their distribution, some researchers (E. C. Cano 2013) proposed to elevate the *discolor* var. to the rank of species as *Glebionis discolor* (d'Urville 1822).

Therefore, a different approach based on molecular data is needed to further investigate the genetic grounds underneath the differentiation of the two varieties.



Figure 3 Co-occurrence of *Chrysanthemum coronarium concolor* and *discolor* varieties in an abandoned field (for geographic reference see Table 1 sampling ID #5)

Heterocarpy and dormancy in Asteraceae

Imbert reported that heterocarpy/heterospermy is known to occur in 18 families of angiosperms, being most common in Asteraceae and Chenopodiaceae (E. Imbert 2002).

Sixty-three and three-tenths percent of the species and 52.5% of the genera belong to the Asteraceae and 8.3 and 10%, respectively, to the Chenopodiaceae. In Asteraceae, fruit heteromorphism, i.e. the difference between central and peripheral achenes (McDonough 1975), may be associated with a difference in germination behaviour, e.g. in *Bidens*, *Senecio*, *Picris* and *Crepis* (McEvoy 1984), (Rocha 1996), (E. E. Imbert 1999), (E. Imbert 2002), (Brändel 2004), (Brändel 2007). Central achenes are often less dormant and germinate over a wider range of temperatures, to higher percentages and at faster rates than peripheral ones (J. B. Baskin 1976), (Forsyth C. 1982), (Corkid 1991) (Rocha 1996) (Brändel 2007).

In seed heteromorphism the different fruit or seed morphs usually have differential ecological behaviour, mainly germination requirements and dispersal abilities. Peripheral achenes are frequently heavier, show more limited dispersal and have stricter requirements for germination (p.e. (E. E. Imbert 1999) (Clavijo 2001). In most of the heterocarpic Asteraceae central achenes germinate immediately when favourable conditions occur while peripheral achenes show delayed germination. Differential dispersal and germination allow spreading offspring in space and time, thus reducing density dependent effects such as sib competition, predation or pathogen attack (D. H. Janzen 1971) (Augspurger 1983) (Levin S. 1984) (Harms 2000). In addition, this different ecological behaviour leads to a risk-reducing or bet-hedging strategy that is advantageous in variable and unpredictable habitats such as Mediterranean biomes and highly disturbed, man-made habitats.

Discerning the differential ecology of achene morphs in heterocarpic weedy Asteraceae is of interest in weed population dynamics and basic weed biology studies, which, in turn, can be useful in designing appropriate weed management practices (Bhowmik 1997).

Seed germination

Chrysanthemum coronarium emergence occurs in autumn and seedlings develop into overwinter rosettes that bolt in January and February. Flowering and fruiting start in late February or March and continue to early summer (F. M. Bastida 2004). Flowers consist of central hermaphrodite disc florets and marginal female ray florets with inferior ovaries (Cockshull 1984). These florets develop two morphologically distinct lignified fruit called cypselsae (hereafter named more merely “achenes”), which are prominently composed by sclerotic cells (Mukherjee 1992). The two morphotypes are distinguishable for their average size, peripheral much bigger than central, and for their shape: one-winged central, and three-winged peripheral achenes (Figure 4).

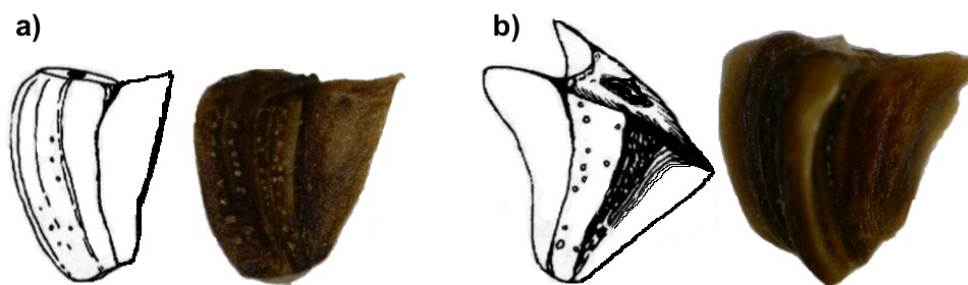


Figure 4 a) One-winged central achene; b) Three-winged peripheral achene.

Both central and peripheral achenes show dormancy in a high proportion, especially peripheral achenes are more dormant than central ones (F. M. Bastida 2004).

Even though some studies (M. P. Chiang 1994) (Vicente 2002) (Hwang 2008) (Banon 2009) (F. J. Bastida 2010) have been investigating the germination behaviour in *Chrysanthemum coronarium*, there is still a bit of uncertainty in defining precisely the type of dormancy of this species.

In a previous study (F. M. Bastida 2004) germination was tested against different conditions: temperature regimes, dark, with a pre-chilling treatment, in presence of GA₃, of nitrate solution, and finally excising seeds from achenes. From the highest germination frequency shown in response to seed excision in presence of the pericarp tissue authors concluded that dormancy in *C. coronarium* is physically imposed by pericarp.

Conversely, in another study (Banon 2009) authors tested *C. coronarium* germinability in presence of several commercial gibberellins and excising seeds from achenes. Interestingly, when excised seeds were irrigated with pericarp leachate this produced an inhibition of germinability (from 92% to 43%), so they concluded that dormancy lies on a combination of physical and chemical dormancy.

Therefore, even though this plant is very common in Mediterranean basin, invasive outside its native environment and produces a large number of seeds, the germination behaviour remains unclear and worthy of study.

Leucanthemum vulgare

Species description and distribution

Leucanthemum vulgare (Vaill.) Lam. (family Asteraceae; tribe Anthemideae; genus *Leucanthemum*) is a monocarpic perennial herb 20 - 40 (100) cm high, with a lignified base, hairless or hairy, simple or branched with a wiggling root-stock where new sprouts originate; flower heads are made up of 15 – 30 white rays that circle a yellow button,

depressed center; florets are clustered in composite heads 2-5 cm wide, with white ray florets radiating out from a yellow centre of disk florets (Pignatti 1982) (Douglas 1998) (Jauzein 2011). Its chorological type is Eurasiatic, Euri-Mediterranean and Euro-Siberian (Pignatti 1982) (Lauber 2001) (Meusel 1965) (Figure 5). In northern Europe flowers bloom from June to August.

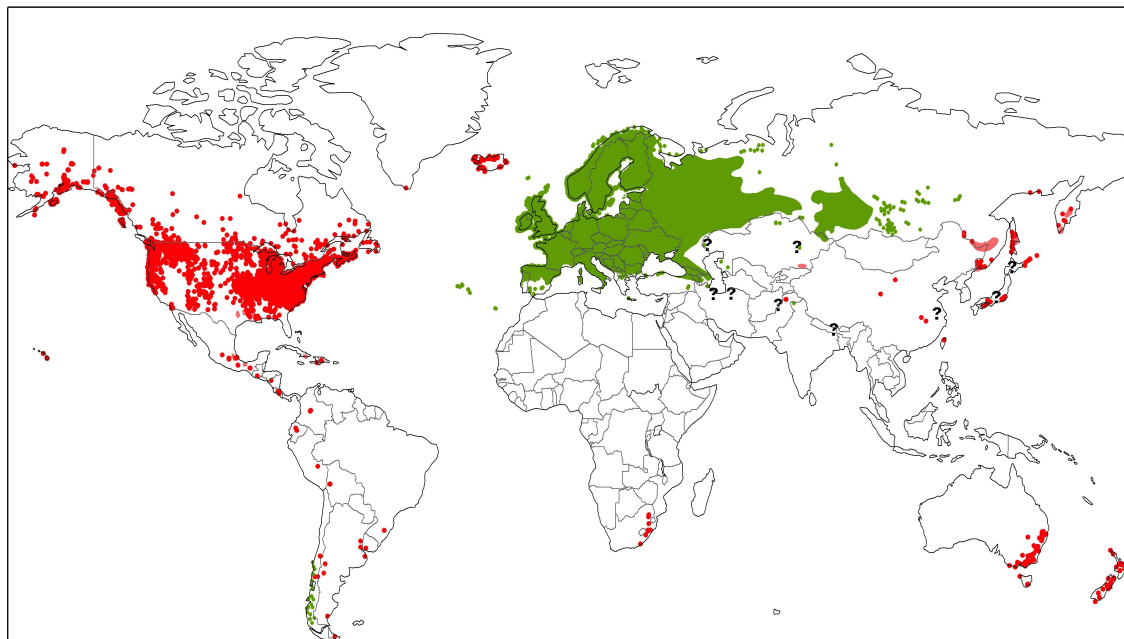


Figure 5 *Leucanthemum vulgare* distribution according to H. Meusel. Figure re-drawn from (Meusel 1965) with the permission of AG Chorology & Macroecology of Vascular Plants, University of Halle. Red dots represents neophytic occurrences, green dots original distribution area.

Leucanthemum vulgare is highly adaptable to a variety of sites. It can grow in coarse to medium textured soils and can be found in moist to moderately dry sites; however, it does prefer abundant sunlight. Once planted as an ornamental, it escaped cultivation and is now common in native meadows, pastures, fields in open and thick woodlands, along waterways and roadsides and it was reported as invasive plants Figure 7 (Khuroo 2010). It is also found in disturbed areas, hay fields, gardens and lawns, and irrigation ditches.

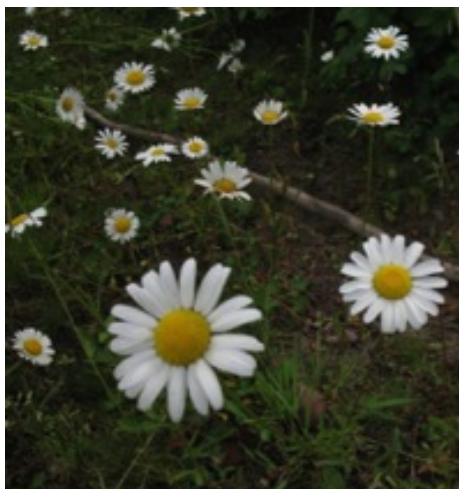


Figure 6 *Leucanthemum vulgare*



Figure 7 A Conifer forest-opening invaded by *Leucanthemum vulgare*

Seed germination

The fruit of *Leucanthemum vulgare* is a small flat seed (Figure 8), dark gray in color with no pappus. One plant can produce over 500 seeds and seeds can remain viable in the seedbank for up to three years.

This plant does not show any dormant behaviour and has a germination temperature between 15 and 20° C and it may germinate either in spring or in early autumn (Keller 1999). Seed germination is inhibited by continuous darkness but otherwise not affected by variation in light (K. R. Thompson 1989).

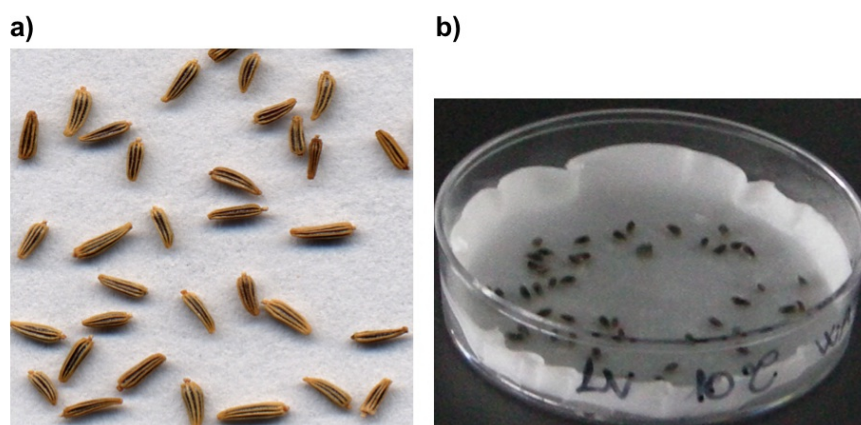


Figure 8 a) *Leucanthemum vulgare* seeds; b) *Leucanthemum vulgare* seeds imbibed in a Petri dish used for germination tests

Varietal genotyping

The assessing of genotypic diversity of closely related taxonomic entities is a challenging goal and its difficulty mainly relies on the reproductive system and species distribution.

Chrysanthemum sensu lato consisted of 27 genera which has been taxonomically placed in the subtribe Chrysantheminae O. Hoffm., the tribe Anthemideae Cass (Shih 1983) while it has been placed in different subtribes in the tribe Anthemideae (Bremer 1993). The main centres of distribution of *Chrysanthemum sensu lato* are two areas: one in the Mediterranean basin and the other in China and Japan, which are considered as ancestors of the Mediterranean species (Dowrick 1952).

The varietal genotyping in *Chrysanthemum* with traditional techniques markers such as morphological and cytological markers was observed to be not effective (Wolff 1994). Therefore, other techniques for identifying molecular markers such as random amplified polymorphic DNA (RAPD) have been developed and applied (Williams 1990). The RAPD technique is more straightforward compared to RFLP analysis and requires only nanograms of genomic DNA. It has been employed in different Brassica species for varietal identification and genetic diversity studies (Jain 1994). However, this technique is sensitive to subtle changes in reaction conditions and hence difficult to reproduce.

Inter simple sequence repeat (ISSR) markers with low cost and low labour requirement but with high reliability have been developed since 1994 (Zietkiewicz E. 1994). Like any other PCR-based marker, it is rapid and require only small amount of the template DNA. ISSR amplification does not require genome sequence information but produce highly polymorphic patterns, and it seem to have the reproducibility of SSR's and the usefulness of RAPD's (Bornet 2001), and thus combine the advantages of SSR and the utility of RAPD. ISSR markers have been used to determine the genetic diversity (Qian 2001). It was also applied to the study of genetic relationships and phylogenetic analysis of Anthemideae tribe (El-Twab A. M. H. 2010).

On the other hand, DNA barcoding is a diagnostic technique for species identification, using a short, standardized DNA region, i.e., the "DNA barcode" (www.barcoding.si.edu) (Lahaye 2008). A DNA barcoding to properly work the sequence variation must be high enough between species so that they can be discriminated from one another; however, it must be low enough within species that a clear threshold between intra- and interspecific genetic variations can be defined. Although the use of DNA

barcoding for identification and taxonomy has been controversial (Ebach 2005) (Will 2005) a growing scientific community has embraced DNA barcoding as a practical tool for biodiversity studies, for example to facilitate inventories of very diverse but taxonomically poorly known regions (Blaxter 2004) (D. H. Janzen 2005).

DNA barcoding in plants is now better established (Hollingsworth 2009) than in the past (Rubinoff 2006) (Pennisi 2007) after the creation of the stable Plant Working Group (http://www.barcoding.si.edu/plant_working_group.html). This consortium approved the usage of *matK* and *rbcL* as the BARCODE regions for Land Plants. So that nowadays, plant barcode resources are catalogued in the Barcode Of Life Database (BOLD) (<http://www.boldsystems.org/>) web portal and available for search for species identification.

Aim of the thesis

The aim of this work was to perform a comparative study on two common Asteraceae weeds, a dormant species, *Chrysanthemum coronarium* var. *concolor* and *discolor*, and a non-dormant one, *Leucanthemum vulgare*, where a secondary dormancy could be induced. In particular, the focus of this research was to determine their germination physiology, and investigating their dormancy release and dormancy induction requirements. Beyond the characterization of germination and dormancy patterns, this study aims to analyse these processes with a molecular approach through isolation of candidate genes associated with dormancy onset and its regulation.

Materials and Methods

Study sampling

Leucanthemum vulgare accession was collected in summer 2010 by Millennium Seed Bank collector team (Figure 9 and Table 1). Seeds were then cleaned and kept at +5° C with controlled RH conditions.

For *Chrysanthemum coronarium* two different seed lots were used in this study: a seed lot harvested in early summer of 2012 and another one harvested in the subsequent year in the same period, see Table 1 and Figure 9. In the older collection only *Chrysanthemum coronarium* var. *concolor* was sampled, while in 2013 field sampling *concolor* and *discolor* varieties were collected.

In 2012 sampling achenes were collected from around 20 healthy plants in a pure ample *concolor* meadow. On the other hand, in the 2013 sampling since the paucity of *discolor* plants, the harvesting was limited to 16 individuals which was the maximum number of *discolor* occurrences. *Discolor* var. was always found together with *concolor* variety. During the early spring 2013 thirty-two flowering plants, sixteen individuals for each variety, were identified and labelled. From each individual an adequate amount of fresh leaves was taken and kept on ice until stored at -80° C for subsequent genotyping analyses.

Plants labelling was carried out for subsequent discrimination during seed dispersal season when is not possible to distinguish the two *C. coronarium* varieties from florets colour. Achenes of the two varieties were collected from marked plants and kept at 15% of Relative Humidity and 15° C before used in germination tests.

Collection ID	Year of collection	Taxon	Height above sea level	City - Country	Latitude	Longitude
1	2010	<i>Leucanthemum vulgare</i>	240 m	Ardingly -England	51° 3'58.40"N	0° 5'41.24"O
2	2012	<i>Chrysanthemum coronarium</i> var. <i>concolor</i>	110 m	Catania – Italy	37°29'25.75"N	14°59'23.63"E
3	2013	<i>Chrysanthemum coronarium</i> var. <i>concolor</i>	529 m	Catania – Italy	37°35'11.79"N	14°59'11.68"E
4	2013	<i>Chrysanthemum coronarium</i> var. <i>concolor</i>	297 m	Catania – Italy	37°33'18.86"N	14°55'52.88"E
5	2013	<i>Chrysanthemum coronarium</i> var. <i>concolor</i>	252 m	Catania – Italy	37°32'49.65"N	14°56'45.60"E
6	2013	<i>Chrysanthemum coronarium</i> var. <i>concolor</i>	320 m	Catania – Italy	37°33'41.28"N	14°58'21.60"E
3	2013	<i>Chrysanthemum coronarium</i> var. <i>discolor</i>	529m	Catania – Italy	37°35'11.79"N	14°59'11.68"E
4	2013	<i>Chrysanthemum coronarium</i> var. <i>discolor</i>	297 m	Catania – Italy	37°33'18.86"N	14°55'52.88"E
5	2013	<i>Chrysanthemum coronarium</i> var. <i>discolor</i>	252 m	Catania – Italy	37°32'49.65"N	14°56'45.60"E
6	2013	<i>Chrysanthemum coronarium</i> var. <i>discolor</i>	320 m	Catania – Italy	37°33'41.28"N	14°58'21.60"E

Table 1 GPS coordinates of sampling sites.



Figure 9 Map of collection of *Leucanthemum vulgare* and *Chrysanthemum coronarium*

Germination experiments

Germination experiments consisted of three replica samples of 50 seeds each. Basing on the different samples size seeds (*L. vulgare*) and achenes (*C. coronarium*) were sowed in two layers of moist filter paper (Whatmann) in plastic Petri dishes, respectively, with 5 cm diameter and with 9 cm diameter. For *Chrysanthemum coronarium* the two morphotypes were always tested separately. Filter paper imbibition was carried out always with double-distilled water when not differently indicated. Since the vast mould proliferation during germination tests, achenes of *Chrysanthemum coronarium* were always washed with 1% of commercial chlorine and then rinsed 3 times in distilled water before sowing them in Petri dishes.

Experiments were conducted in temperature (± 1 °C) and light ($40 \mu\text{mol m}^{-2} \text{s}^{-1}$) controlled conditions using a 12 h or 8/16 h daily thermo- (for alternating temperatures) and photo-period (= light hereafter). In the incubators actual temperature were checked repeatedly. Germination was defined as radicle emergence from the testa by at least 1 mm often with a small radicle flexing. All emerging seedlings were counted and removed from the dish. At the end of the experiments (upon full germination, or after 2 weeks without any further

germination) cut tests determined the number of un-germinated but viable seeds; defective seeds (i.e. empty, damaged and infected) were excluded from the all calculations.

In *Chrysanthemum coronarium* seed germination was observed to occur always after the fruit coat rupture, as a preparatory event that precedes germination, hence, FCR was recorded and used for subsequent data elaborations.

For climatic data of the collecting sites and to choose adequately the germination temperatures to use World Climate (<http://www.climate-charts.com>) was used as climatic reference database.

Seed excision

To observe the germination behaviour in *C. coronarium* without pericarp tissue, seeds were excised, using a razor blade, from both peripheral and central achenes and sowed at 5, 10, 15, 20, and 25° C with 12h light. For this test both seeds lots were used (2012 and 2013) to compare seeds viability. Once deduced the optimum temperature range, germination of excised seed was tested in alongside pericarp tissue to observe any possible chemical inhibitory effect.

Water uptake

In order to test the presence of physical dormancy in *C. coronarium* water uptake of intact achenes was monitored. Achenes were, hence, imbibed at 10° C and weighed with a 4-place precision balance at regular intervals of time until reaching a stable plateau. Isolated seeds were also included in this test for comparison.

Anatomical analyses of pericarp tissue

To observe the anatomical structures in the *Chrysanthemum coronarium* pericarp a tissue dissection was carried out in both achene morphotypes by using a razor blade and visualizing tissues with a microscope (Primo Star - Carl Zeiss Microscopy) using different magnifications (10X, 40X and 100X).

Germination in dark

In order to test whether *Chrysanthemum coronarium* germination is dependent on the presence of light or not, achenes from 2013 collection were tested in continuous darkness at 5, 10, 15, 20 and 25° C for 12 weeks in the dark and checked once at the end of the test. Darkness was achieved by wrapping Petri dishes in 2 aluminium foil layers and kept inside

a thick-textured black bag for the duration of the experiment. In the meantime a control experiment was carried out in light presence.

Cold stratification

A chilling stratification to mimic natural conditions during winter was carried out in *Chrysanthemum coronarium* in order to observe its impact in dormancy release. Cold stratification was carried out sowing achenes for 0, 4, 8 and 12 weeks at 5° C in darkness (see germination in darkness for methodology), and transferring them at 20, 25, 30, and 35° C after stratification.

Dry storage

Chrysanthemum coronarium central achenes from the 2012 collection were used for a long-term dry storage test that was carried out keeping achenes at 10-22° C with a Relative Humidity between 20 and 30% up to 500 days. Achenes germination were tested after 80, 200, 260 and 500d and compared with seed germination of fresh achenes.

Dry after ripening

Achenes from the 2013 collection of *Chrysanthemum coronarium* were used to test the effect of dry after-ripening treatment on seed dormancy. Hot and dry native environment summer climatic conditions were simulate keeping achenes for 0, 4, and 12 weeks at alternating temperature of 35°/20° C (night/day) with a 8/16 photoperiod with a Relative Humidity of, respectively, 35% and 58%. Afterwards achenes were imbibed at 5, 10, 15 and 20° C.

Wet warm stratification

Wet warm stratification was tested in achenes from the 2013 collection of *Chrysanthemum coronarium* to analyse the different germination response in comparison with cold stratification. Thus, achenes were imbibed at 35/20° C with 8/16 hours of photoperiod. This treatment was performed for 3, 5 or 8 weeks and after which achenes were transferred to germination temperatures 5, 10, 15 and 20° C.

Seed osmo-priming

To determine the effect on germination behaviour of seed osmo-priming treatment in *Leucanthemum vulgare* seeds were primed imbibing them with Polyethylene-glycol (PEG)

(concentration and to have an osmotic potential of -0.072 MPa) and incubated at 5° C for 1, 5, and 9 weeks. Osmo-priming at 10° C were carried out with an osmotic potential of -0.069 MPa for 1, 5, and 9 weeks. Light role in affecting germination response during priming was also tested imbibing seeds in continuous darkness for 1 week. Both for light and darkness experiments, after priming treatment seeds were washed with tap water and imbibed in double distilled water at 10, 15, 20, 25, and 30° C.

Seed hydro-priming

Leucanthemum vulgare seeds were hydro-primed by soaking seeds in water or with different osmotic potentials: -0.02, -0.072, -0.292 MPa. Afterwards seeds were washed with tap water and imbibed in double distilled water at 10, 15, 20, 25, and 30° C.

ABA synthesis

In both *Chrysanthemum coronarium* and *Leucanthemum vulgare* the ABA biosynthesis was investigated to observe its role in dormancy maintenance. To do that seeds (*L. vulgare*) or achenes (*C. coronarium*) were imbibed with different concentration 0.03, 0.1, 0.3, 1, 3, 10, 30, 50, and 100µM of fluridone (1-methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]-4(1*H*)-pyridinone) which inhibits the carotenoid pathway depleting the ABA biosynthesis (Lem 1981).

Incubation in agitated water

In *Chrysanthemum coronarium* achenes were washed in 200 ml water for 24 h on a rotating shaker to remove endogenous ABA as published in (M. T. Le Page-Degivry 1990). Afterwards, soaked achenes were used to run simultaneously two different tests: *i*) achenes were imbibed in double distilled water and incubated at 20° C; *ii*) achenes were imbibed with a 100µM solution of fluridone and incubated at 20° C. In the first test, water washing was done to remove the ABA already present in the achene, while in the second test, in addition to removing the pre-existing ABA, fluridone was added to prevent any further ABA biosynthesis during the achene imbibition.

Giberellic acid

After the osmo-priming treatment, *Leucanthemum vulgare* seeds appeared to be inducted in a non-deep physiological dormancy status. Thus, in order to investigate the degree of physiological dormancy, osmo-primed seeds were imbibed with giberellic acid at different

concentrations 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 μ M. control experiment was carried out by imbibing seeds in double distilled water.

Statistical analyses

Germination results statistical support was carried out testing normality and homogeneity of data, and subsequently the relative analysis of variance (Anova). All the elaborations were carried out by using R program (R Development Core Team, 2008).

Molecular analyses

Chrysanthemum coronarium varietal genotyping

To investigate the genetic relationship between the two varieties of *Chrysanthemum coronarium*, two different approaches were carried out: an intra-specific analysis using markers which produce highly polymorphic patterns to evaluate their genetic diversity; an inter-specific approach to investigate whether the genetic diversity degree arises from differences at the species rank or not. In the first system the profiles of Inter Simple Sequence Repeats (ISSRs) were used, and in the second one ribulose-bisphosphate carboxylase gene (rbcL) and maturase K (mat-K) gene were sequenced on both strands.

DNA extraction

For each of the 32 individuals (16 *concolor* + 16 *discolor* var.) around 90 mg of fresh leaf tissue was used to extract DNA following the CTAB/Chloroform-Isoamyl alcohol protocol published by (J. J. Doyle 1987) (J. J. Doyle 1987) (Cullings 1992).

Even though the presence of 4% Polyvinylpyrrolidone (PVP) in the extraction buffer the polyphenolic compounds exerted an inhibitory effect on the Taq polymerase activity during the amplification DNA. Thus, a final concentration of 2% of Polyvinylpyrrolidone was added to the PCR mix (Koonjul 1999) to have reproducible and stable DNA amplifications. From DNA extraction 25 ng of DNA was used as template for subsequent PCR analyses.

ISSR profiling

Internal Sequence Simple Repeats (ISSR) PCR were carried out for all the samples in a total volume of 25 μ L containing 25 ng of DNA template, 2.5 μ L of 10 \times PCR buffer (100 mM Tris-HCl, 15 mM MgCl₂, 500 mM KCl), 2% of PVP, 0.5 μ L of dNTPs (10 mM each), 0.5 μ L of primer (10 mM), 1.5 unit of Solis HotStart Taq DNA polymerase (Solis BioDyne, Tartu, Estonia).

Twenty-one ISSR markers, 3' or 5' anchored primers (synthesized by Eurofins MWG Operon, Ebersberg, Germany), were tested and 9 of them were selected basing on their band pattern and reproducibility (see Table 2).

Primer ID	Primer sequence 5'-3'	Selected for genotyping	Annealing temp. (° C)	Source of publication
Cc-01	ACCACCACCACCACCACCG	Y	48	(Dogan 2007)
Cc-02	GAGAGAGAGAGAGAGAGAC	Y	48	(Dogan 2007)
Cc-03	AGAGAGAGAGAGAGAGAGC	Y	50	(Dogan 2007)
Cc-04	ACACACACACACACACACG	Y	50	(Dogan 2007)
Cc-05	ACACACACACACACACCG	Y	50	(Dogan 2007)
Cc-06	ACACACACACACACACCY	N	50	(Dogan 2007)
Cc-07	CAGCACACACACACACACA	N	50	(Dogan 2007)
Cc-08	CGTCACACACACACACACA	N	50	(Dogan 2007)
pBr_01	GAGCAACAACAACAACAA	N	50	(Huangfu 2009)
pBr_02	CTGGTGTGTGTGTGTGTGT	N	50	(Huangfu 2009)
pBr_03	AGAGAGAGAGAGAGAGGTG	Y	50	(Huangfu 2009)
pBr_04	GAGAGAGAGAGAGAGAACC	Y	50	(Huangfu 2009)
pBr_05	AGAGAGAGAGAGAGAGYC	Y	50	(Huangfu 2009)
pBr_06	CTCTCTCTCTCTCTCTRA	N	50	(Huangfu 2009)
pBr_07	DBDACACACACACACAC	N	50	(Huangfu 2009)
pBr_08	HVHTGTGTGTGTGTGTGTG	N	50	(Huangfu 2009)
He-816	CACACACACACACACAT	N	50	(Smitsen 2003)
He-821	GTGTGTGTGTGTGTGTGTT	N	50	(Smitsen 2003)
He-819	GTGTGTGTGTGTGTGTGTA	N	50	(Smitsen 2003)
Hy_18	GTGCTCTCTCTCTCTCTC	N	50	(Barcaccia 2006)
Hy_39a	AGCAGCAGCAGCAC	Y	50	(Barcaccia 2006)

Table 2 Primers sequences used for varietal genotyping in *Chrysanthemum coronarium*

The amplification reactions were carried out using a Quanta biotech SI-96 Thermal Cycler (Quanta Biotech Ltd, Surrey, UK) with the following PCR profile: 95° C for 15 min; 35 cycles at 94° for 30 sec, specific annealing temperature for each primer (Table 2) for 1 min, 72° C for 1 min; and final extension at 72° C for 10 min.

Ten µl of PCR products were electrophoresed on 2.0% agarose gels during 2.0 h at 100 V in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.4) and a 100 base-pair ladder (100 bp DNA Ladder, Solis BioDyne, Tartu, Estonia) was used as molecular size marker. Gel was stained for 30 min with 1X Gel Red (© Biotium, USA) and then visualized and photographed using a gel documentation system (BioDoc-It® Imaging System, CA).

***rbc-L* gene amplification and sequencing**

The entire *rbcL* gene was amplified using the “1f” and “1369 r” primers as described in (Olmstead 1992) in a total volume of 70 µL containing 25 ng of DNA template, 7 µL of 10 × PCR buffer (100 mM Tris-HCl, 15 mM MgCl₂, 500 mM KCl), 8.4 µL of MgCl₂ (25

mM), 2% of PVP, 1.4 µL of dNTPs (20 mM each), 1.75 µL of primer (10 mM), 2 unit of Solis HotStart Taq DNA polymerase (Solis BioDyne, Tartu, Estonia).

Amplifications were carried out in the thermal cycler aforementioned with the following PCR profile: 95° C for 15 min; 35 cycles at 94° for 30 sec, 1 min at 48°, 72° C for 1 min; and final extension at 72° C for 10 min. The obtained PCR products were purified and sequenced on both strands by Macrogen Inc. (Netherlands) using PCR primers. Sequencing was conducted under BigDye™ terminator cycling conditions and products purified using ethanol precipitation and run using automatic sequencer ABI-3730XL.

mat-K gene amplification and sequencing

The central region of mat-K gene was amplified using the “390f” and “1326 r” primers as described in (Schaefer 2011) in a total volume of 70 µL containing 25 ng of DNA template, 7 µL of 10 × PCR buffer (100 mM Tris–HCl, 15 mM MgCl₂, 500 mM KCl), 8.4 µL of MgCl₂ (25 mM), 2% of PVP, 1.4 µL of dNTPs (20 mM each), 1.75 µL of primer (10 mM), 2 unit of Solis HotStart Taq DNA polymerase (Solis BioDyne, Tartu, Estonia).

Amplifications were carried out in the thermal cycler aforementioned with the following PCR profile: 95° C for 15 min; 35 cycles at 94° for 30 sec, 1 min at 48°, 72° C for 1 min and 30 seconds; and final extension at 72° C for 10 min. The obtained PCR products were purified and sequenced on both strands by Macrogen Inc. (Netherlands) using PCR primers. Sequencing was conducted under BigDye™ terminator cycling conditions and products purified using ethanol precipitation and run using automatic sequencer ABI-3730XL.

Data analysis

ISSR Band analysis was carried out using Total Lab 100 (TotalLab Ltd, UK) and only non-overlapping and highly reproducible bands with a molecular size comprised from 350 to 1700bp were considered for fragments detection.

Genetic diversity was measured by the percentage of polymorphic bands (P), which was calculated by dividing the number of polymorphic bands at population and species levels by the total number of bands surveyed. Shannon indices of diversity, namely both the total diversity (Hsp) and the intra-population diversity (Hpop), were also calculated using the computer program POPGENE software package Version 1.32. Principal Coordinates Analysis was carried out with GeneAlex program (Peakall, 2006)

Sequences of rbc-L and mat-k genes were blasted against BOLD databases (<http://www.boldsystems.org/>) (Ratnasingham 2007) for confirmation. Sequence alignment

was carried out using CLUSTAL W algorithm (Larkin 2007), and phylogenetic elaboration was carried out with PAUP program (Swofford, 2003).

Identification and isolation of reference and dormancy associated genes in *C.*

coronarium* and *L. vulgare

Reference genes

Quantifying gene expression levels through reverse transcription–quantitative real-time PCR (RT–qPCR) is the preferred method for targeted gene expression measurements. However, normalization, is necessary to correct for sample input and reverse transcriptase efficiency, is a crucial step to obtain reliable RT–qPCR results. Stably expressed genes (i.e. genes whose expression is not affected by the treatment or developmental stage under study) are indispensable for accurate normalization of RT–qPCR experiments. Lack of accurate normalization could affect the results and may lead to false conclusions.

Some of the house keeping genes involved in basic cellular activities such as *18s rRNA*, *25s rRNA*, *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH) and *ubiquitin* (UBQ) are some of the commonly used internal control genes as they are likely to be expressed at constant levels regardless of experimental conditions (Schmittgen 2000) (Czechowski 2005).

Therefore, selected genes from literature (Dekkers 2011) were used to find their homologous gene family for Asteraceae consulting “Nucleotide” dataset of “NCBI” database. Afterwards, each matched sequences was searched at the *Arabidopsis* electronic fluorescent pictographic (eFP) browser (<http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi>) (Schmid 2005) (Kilian 2007) (Winter 2007) to check how much stable is the expression of the selected gene compared with the treatment as reported in the browser. Most stable gene sequences, hence, were aligned for cloning primers designing to use in downstream *C. coronarium* and *L. vulgare* PCR amplifications.

Dormancy associated genes

On the basis of the specific literature on molecular regulation of seed dormancy (Liu 2007) (G. C. Chiang 2009) (L. H.-L.-B. Bentsink 2010) (S. D.-S.-S. Footitt 2011) *DOG1* (*DELAY OF GERMINATION 1*), *FLC* (*FLOWERING LOCUS C*), *HUB2* (*HISTONE MONOUBIQUITINATION 2*) genes were included. In addition the *EXP Expansin* gene was selected for his role in mediating cell expansion during radicle elongation (F. D. Chen 2001).

The overall workflow used for marker selection is illustrated in Figure 10.

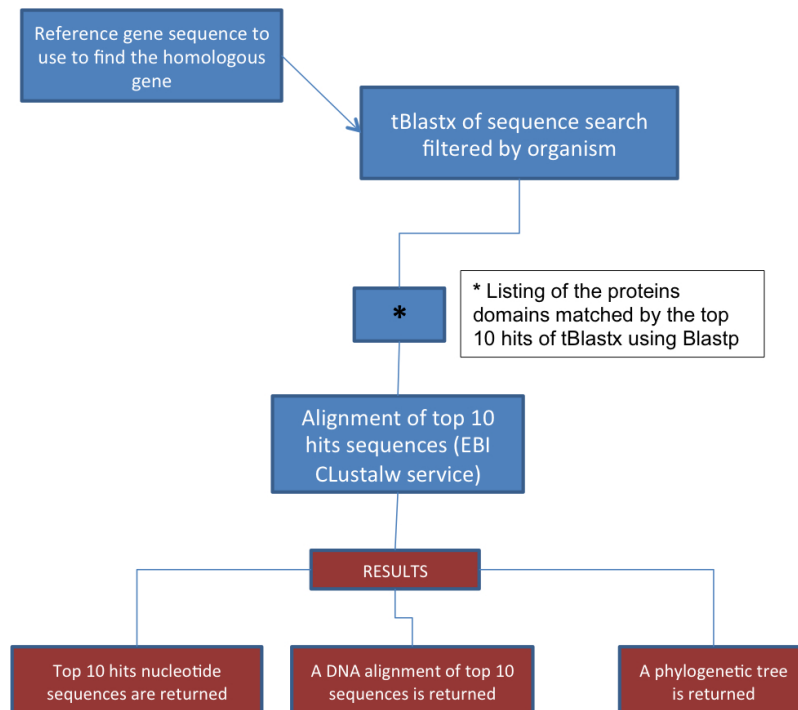


Figure 10 Workflow for primer designing of dormancy associated genes in *C. coronarium* and *L. vulgare*

DNA extraction

Fifty seedlings of *Leucanthemum vulgare* about 1 cm long each along with their seed coat were grounded in a mortar pestle using liquid nitrogen for DNA extraction. A CTAB/Chloroform-Isoamyl Alcohol procedure was used for DNA extraction as in (Kocik 1996). Samples were diluted 1000 times, shared through vigorous vortexing to make DNA more accessible for downstream amplification purposes.

Both *L. vulgare* and *C. coronarium* (herein for this chapter just *concolor* variety, DNA extracted following method described at the beginning of this chapter) extracted DNAs were used as templates for amplification of reference genes and dormancy-associated genes.

RNA extraction

Total RNA was isolate from different stage of dormancy of *L. vulgare* seeds: dry seeds (as reference control), 1 week primed seeds at 5° C, 1 week primed seeds at 10° C, 5 weeks primed seeds at 5° C, 5 weeks primed seeds at 10° C, and after imbibition upon germination (endosperm rupture and when upon radicle protrusion not longer then 1 mm).

Each sample was made up by three replicas of 50 seeds each. RNA was extracted using the hot borate protocol as in (Wan 1994) (Toorop 2005). Pellets were dried over-night using a freeze-dryer (Maxi Dry Lyo, Gemini BV) and, afterwards, resuspended in 50 µl of RNA-secure™ (Ambion®, Life Technologies) to inactivate RNases. RNA was quantified with a spectrophotometer WPA Lightwave (Biochrom, Cambridge, UK). Half µg per each sample was loaded in a electrophoresis gel well, run at 100 V in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.4) for 1 hour and visualized through a Gene Genius (Syngene, Synoptics Ltd) bio imaging system.

cDNA first strand synthesis

One µg of total RNA was used as a template for first cDNA strand with Transcriptor First Strand cDNA Synthesis kit (Roche, UK), using anchored-oligo(dT)₁₈ primers. First strand polymerization was carried out in PCR tubes using a thermal cycler DNA Engine Dyad, Peltier Thermal Cycler. Synthesized cDNA was used as template for amplification of reference genes and dormancy-associated genes.

PCR products cloning

Three µL of PCR products were used for ligation with a TA cloning vector, pGEM®-T easy Vector kit (Promega), following the manufacturer instructions with some modifications: 1 µL of ligation reaction was used for transformation and LB medium (for 1 L: 10 g BactoTryptone, 5 g of BactoYeast, and 5 g NaCl) was used. Bacteria were grown in a 1.5 mL polypropylene tube for 1.5 h and 100 µL were plated in a LB/ampicillin plate and incubated at 37° C over-night. Single colonies were picked up with wooden sticks and rinsed with double distilled sterile water in an Eppendorff tube. Ten µL were used as template for PCR colony using plasmid specific primers to amplify the inserted DNA fragment. Electrophoretic gel runs were carried out for evaluating the size of the incorporated fragment and comparing it with the supposed gene length as observed during homologous gene alignment.

When positive colonies were found bacteria were picked up from the twin colony and grown over night at 37° C in 3 mL of LB-medium containing the ampicillin antibiotic. Isolation of plasmid DNA was carried out using the High Pure Plasmid Isolation kit (Roche) starting from a 3 mL of cell culture (transferring two times 1.5 ml to an Eppendorff tube and centrifuging at 14000 rpm for 30 sec). Isolated plasmid DNA samples were quantified using a spectrophotometer (see above for instruments specifications), checked

through inserted fragment amplification, and sequenced by University College London sequencing service.

Sequences analysis

Plasmid sequences were trimmed against vector database to remove any contaminations and then blasted using BLASTn algorithm (NCBI) against Asteraceae dataset sequence or if no results were returned enlarging the dataset including more plant groups. The most similar sequences resulted in homologous genes and the most similar sequences were aligned using CLUSTAL W algorithm (Larkin 2007). This procedure gave an evidence of the origin of isolated sequences (Figure 11).

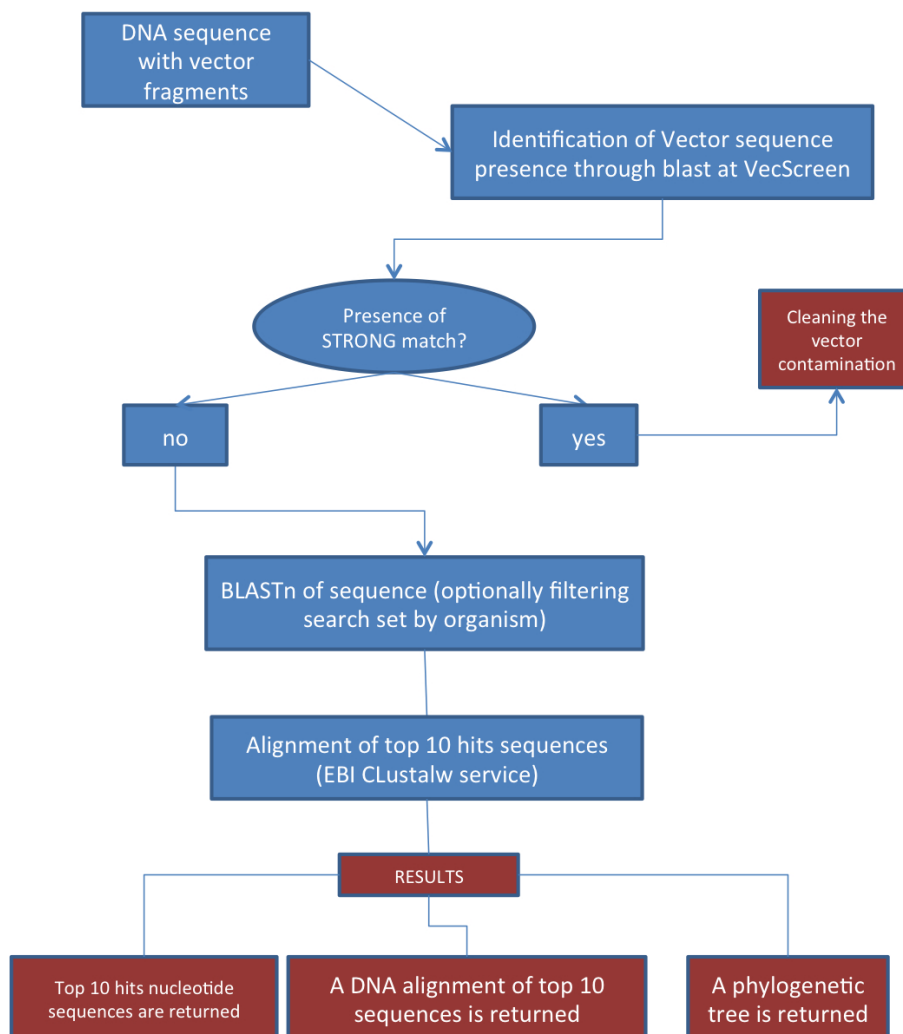


Figure 11 Sequence annotation workflow

Results

Physiology of germination of *Chrysanthemum coronarium*

Germination behaviour

Chrysanthemum coronarium germination of fresh achenes in the two varieties (see Figure 12 and Figure 13) showed a very low response (under 5 %). Radicle emergence always occurred in achenes with an evident rupture in the fruit coat, but not all the ruptured achenes germinated. Higher temperatures produced a significantly increase in FCR events then lower temperatures.

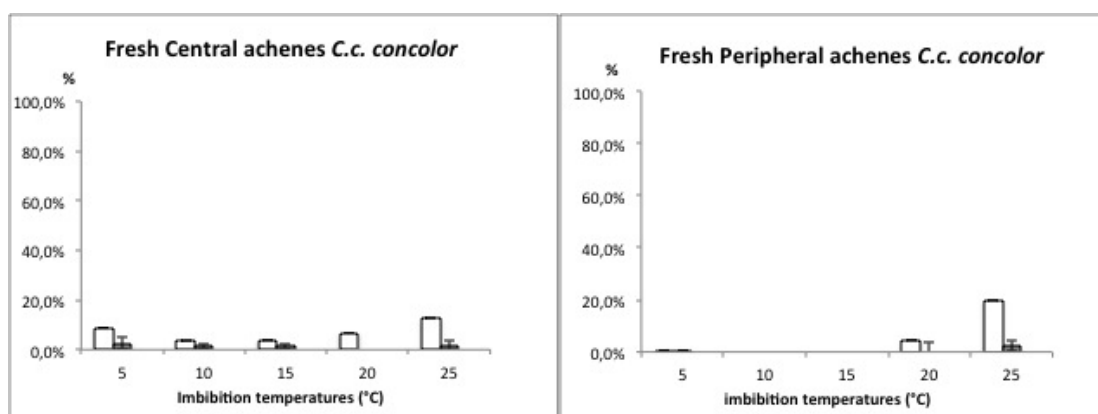


Figure 12 *Chrysanthemum coronarium* var. *concolor* germination at constant temperatures. Fruit coat rupture: open histograms; Seed germination: solid histograms.

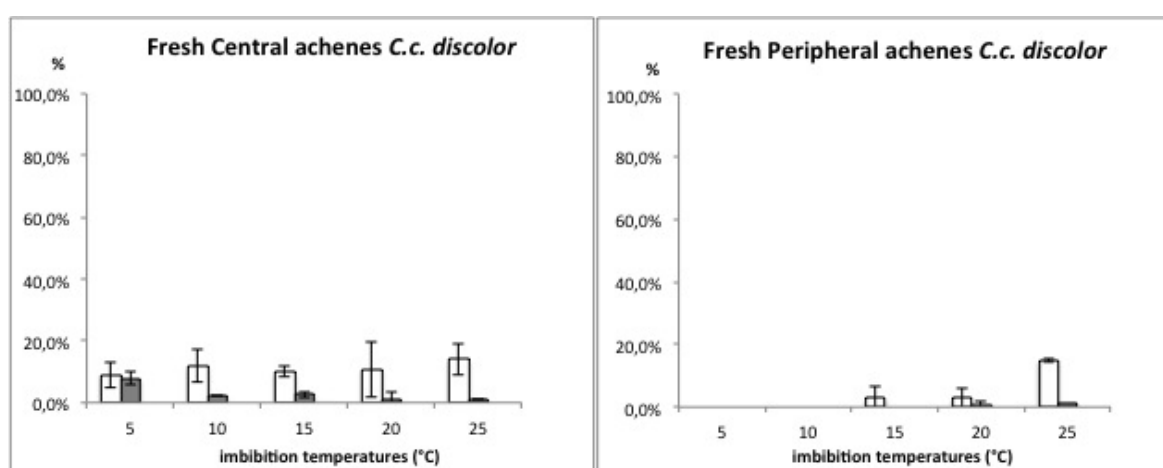


Figure 13 *Chrysanthemum coronarium* var. *discolor* germination at constant temperatures. Fruit coat rupture: open histograms; Seed germination: solid histograms.

Seed excision

Excising seeds from pericarp resulted in very high germination frequency (100%) for both peripheral and central morphotypes (Figure 14). Values on germination speed (t-50) indicated a slower response at low temperatures (Figure 14).

On the other hand, intact achenes germinated fewer than 5% in all the tested temperatures.

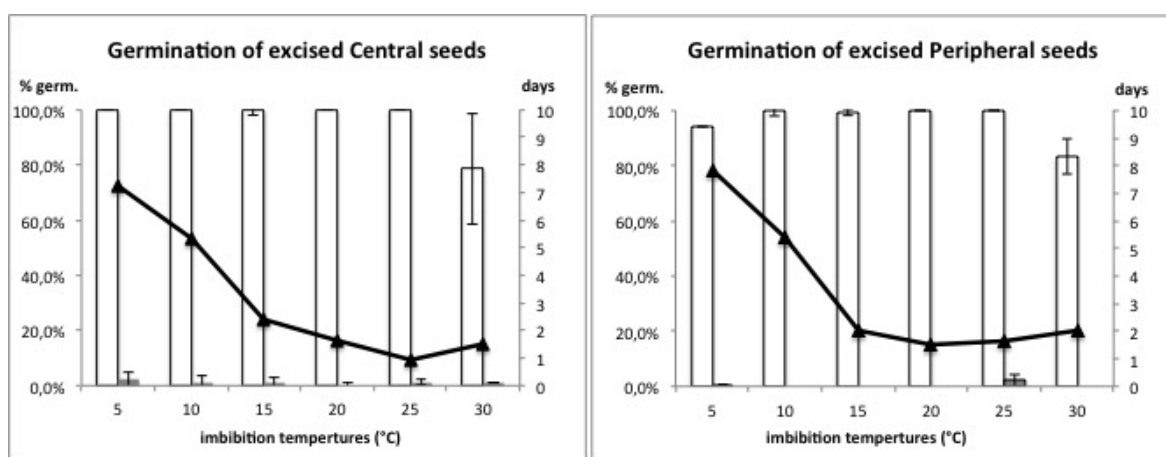


Figure 14 Germination of central and peripheral excised seeds of *Chrysanthemum coronarium* var *concolor*. Histograms follow principal axis (final germination %), while curve follows the secondary axis (number of days until 50% of final germination). Open histograms: seed germination of excised seeds. Solid histograms: seed germination of intact achenes. Solid line: excised seed germination t-50.

Furthermore, sowing excised seeds in close contact with pericarp tissue did not showed any significant inhibitory effect (Figure 15).

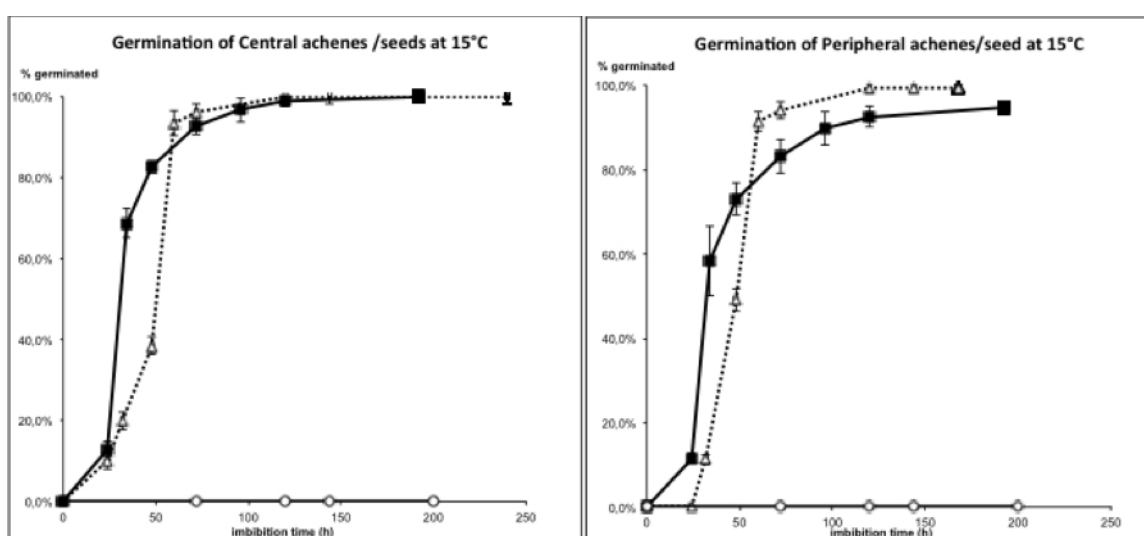


Figure 15 Germination at 15° C of central and peripheral excised seeds of *Chrysanthemum coronarium* var *concolor* in close contact with pericarp tissue; Open circle: intact achenes (control experiment); Solid line: excised seeds with pericarp tissue; Open triangles: excised seeds without pericarp tissue.

Comparison of the germination behaviour of the two varieties

The germination frequency of the two varieties of *Chrysanthemum coronarium* was compared (Figure 16) and statistical support was tested. Normality and homogeneity were respected and Anova analysis indicated no significant differences within the germination behaviour of the two varieties (see Table 3).

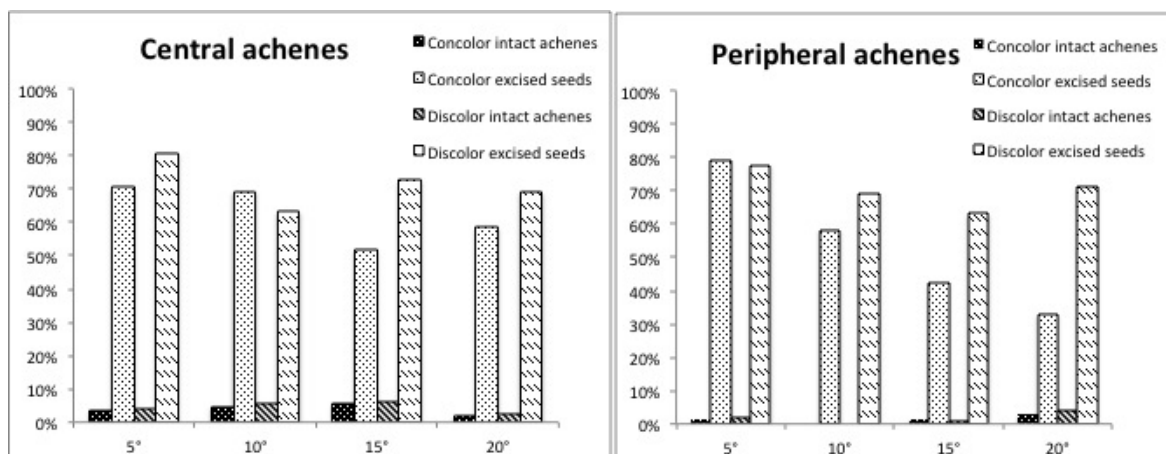


Figure 16 Final germination of intact cypselae (represented with dark bars) and excised seeds (light bars)

	Variety	Morphotype	Pearson test p-value	Bartlett test p-value	Anova test p-value
Intact cypselae	concolor	Central	1	0.9383	0.5265
	discolor		1		
Intact cypselae	concolor	Peripheral	1	0.5999	0.8373
	discolor		0.1573		
Excised seeds	concolor	Central	1	0.7476	0.1728
	discolor		1		
Excised seeds	concolor	Peripheral	0.1573	0.07621	0.1561
	discolor		0.1573		

Table 3 Homogeneity (Pearson's test), normality (Bartlett's test), and analysis of variance (ANOVA); P-value >0.05 null hypothesis is accepted = not significant difference; P-value <0.05 null hypothesis is rejected = significant difference.

Water uptake

Central and peripheral excised seeds showed an increase of weight after imbibition in water and reached a plateau after 48h. This trend is clearly visible in imbibed intact achenes as well (Figure 17). The comparison of relative weights during imbibition showed a more pronounced increase in peripheral excised seeds weights than in central ones.

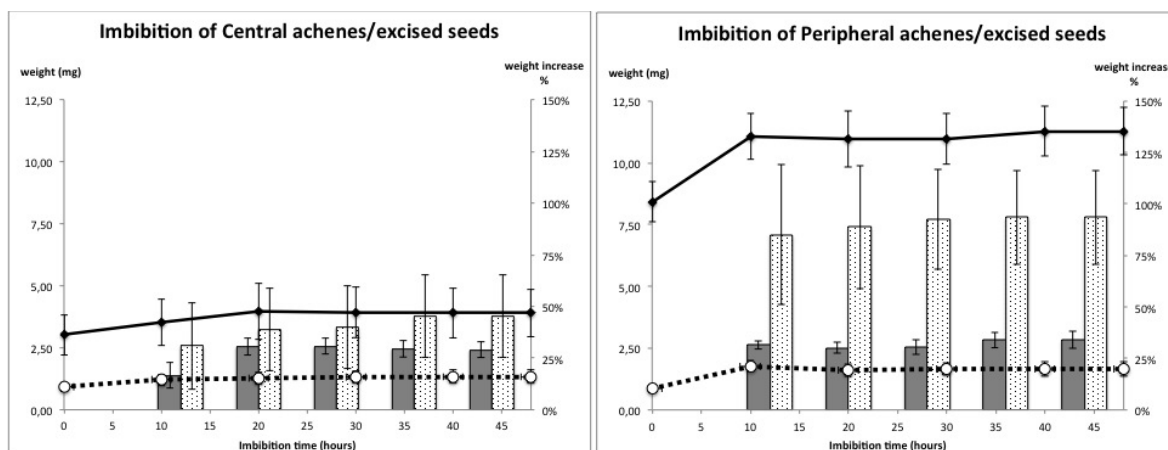


Figure 17 *Chrysanthemum coronarium* var. *concolor* seed imbibition time course. Weight (mg) in principal axis, weight increase (%) in the secondary axis. Curves follow principal axis, while histograms follow secondary axis. Intact achenes are represented by solid line and solid histograms. Excised seeds are represented by dotted lines and dotted histograms.

Anatomical analyses of pericarp tissue

Anatomical analyses conducted on pericarpic tissue in both achene morphotypes (Figure 18 and Figure 19) unveiled the presence of *brachysclereids* cells in mesocarp. The presence of various pit canals in these structures can allow water flow during achene imbibition (Figure 20).

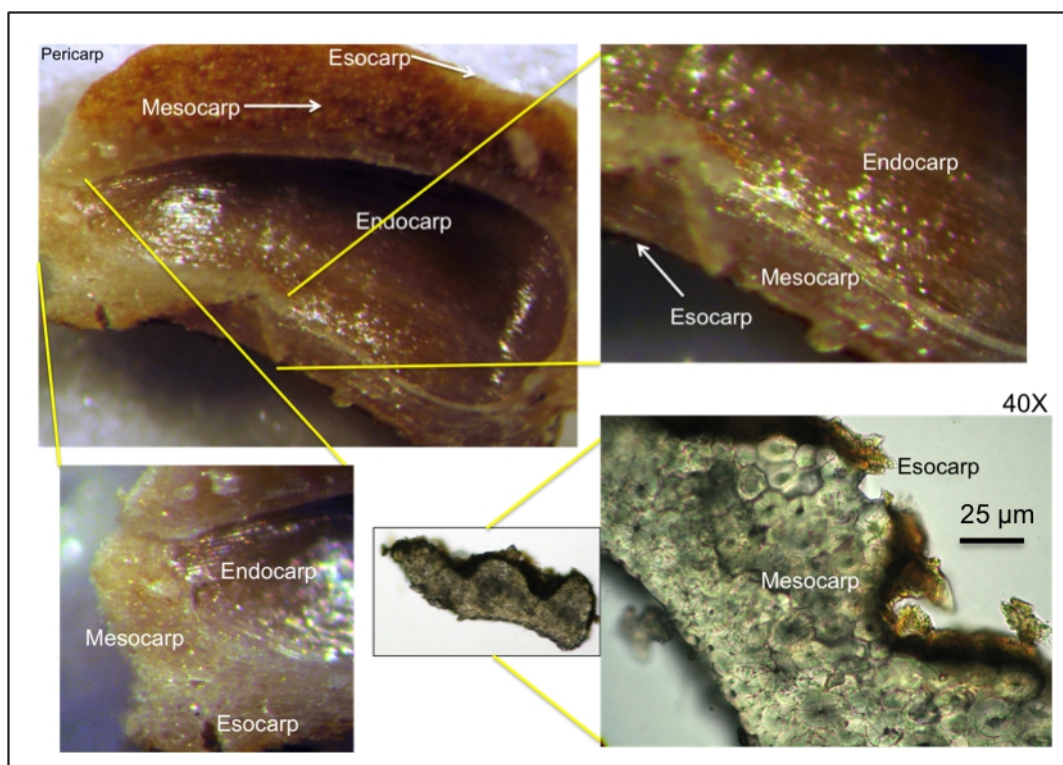


Figure 18 Anatomy of central achene pericarp. On the bottom left a 40X magnification of a cross section of pericarp where sclerotic cells with pit canals are distinguishable.

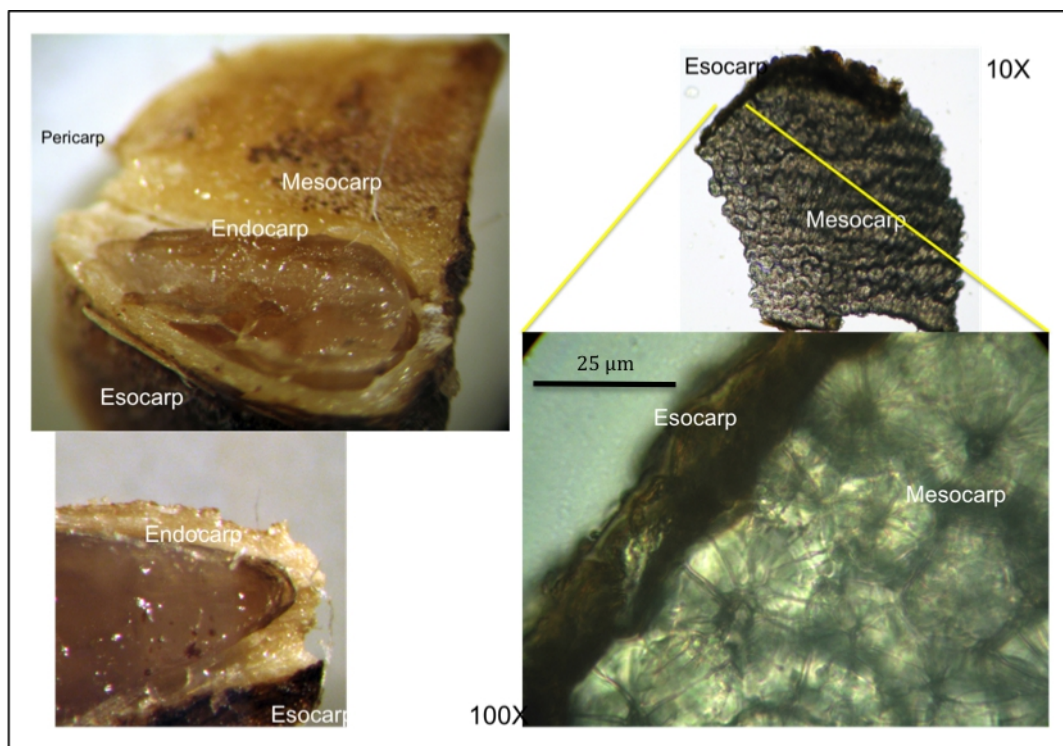


Figure 19 Anatomy of peripheral achene pericarp. On the bottom left a 100X magnification of a cross section of pericarp where sclerotic cells with pit canals are distinguishable.

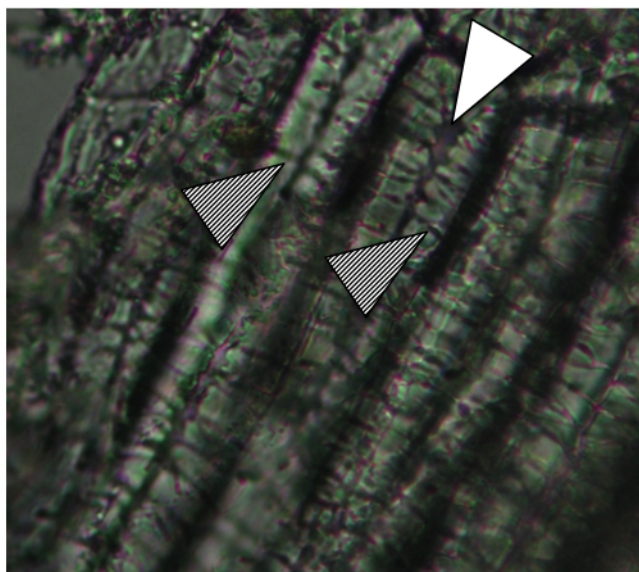


Figure 20 A 100X magnification of a longitudinal section of pericarp. Thick-walled lignified brachysclereids with cell lumen (white arrowhead) and pit canals (striped arrowhead) some of which are branched.

In the following chapters an investigation on the release of physiological dormancy is undertaken.

Germination in dark

The presence of light seems not be significant in germination promotion for both morphotypes. Beyond light or dark conditions higher temperatures results in higher FCR values.

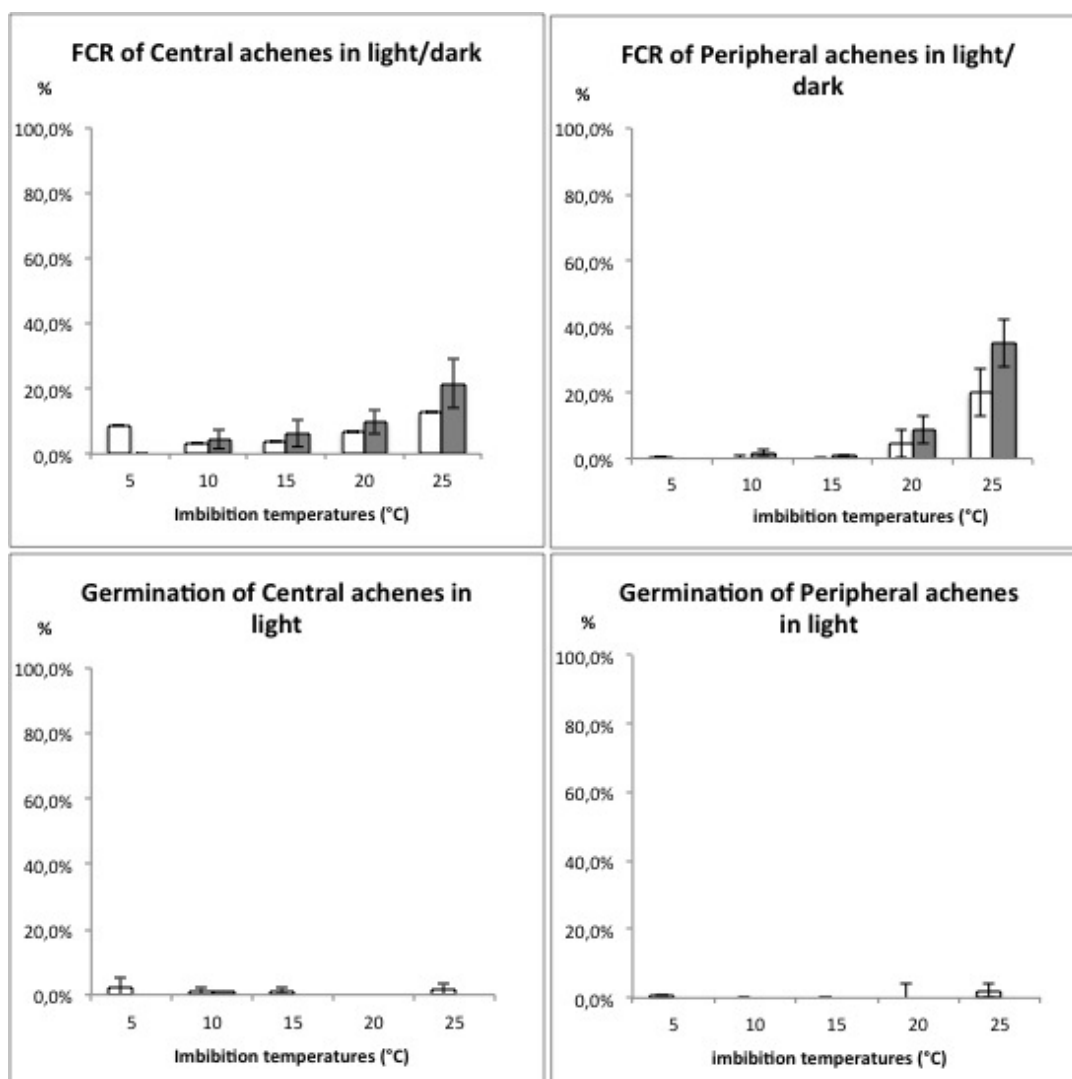


Figure 21 *Chrysanthemum coronarium* var. *concolor* germination in darkness. Fruit coat rupture on top of the figure and below germination frequency. Solid histograms: achenes imbibed in darkness; open histograms: achenes imbibed in light.

Cold stratification

For both morphotypes, at the end of cold stratification no germination neither fruit coat rupture was observed. Fruit coat rupture trend followed the temperatures gradient being more pronounced at higher temperatures with no significant variations respect to the control experiment. Increasing cold stratification time period incubation did not produced any significant growth in germination results.

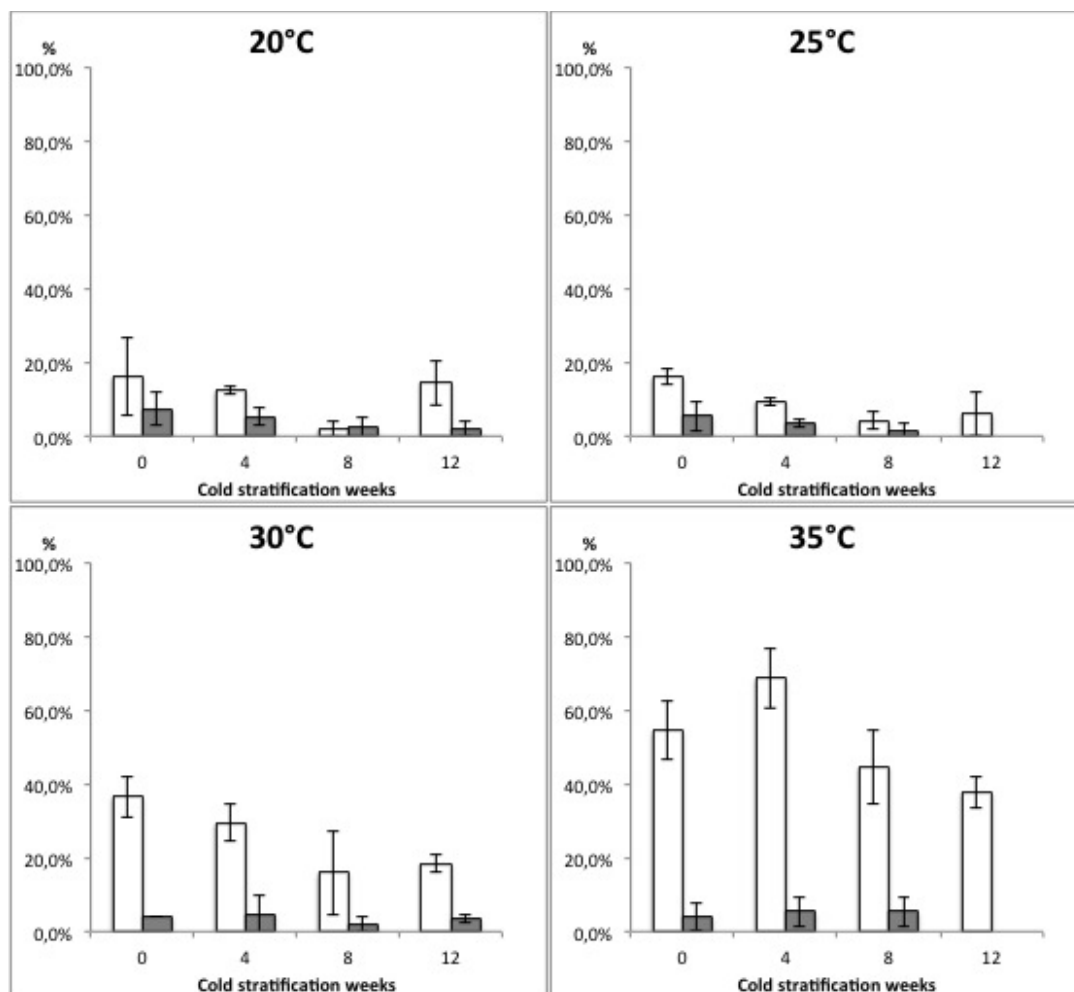


Figure 22 *Chrysanthemum coronarium* var. *concolor* central achenes were stratified at 5° C in darkness for 4, 8 or 12 weeks before imbibition at germination temperatures (20, 25, 30, and 35° C). Fruit coat rupture: open histograms; Seed germination: solid histograms.

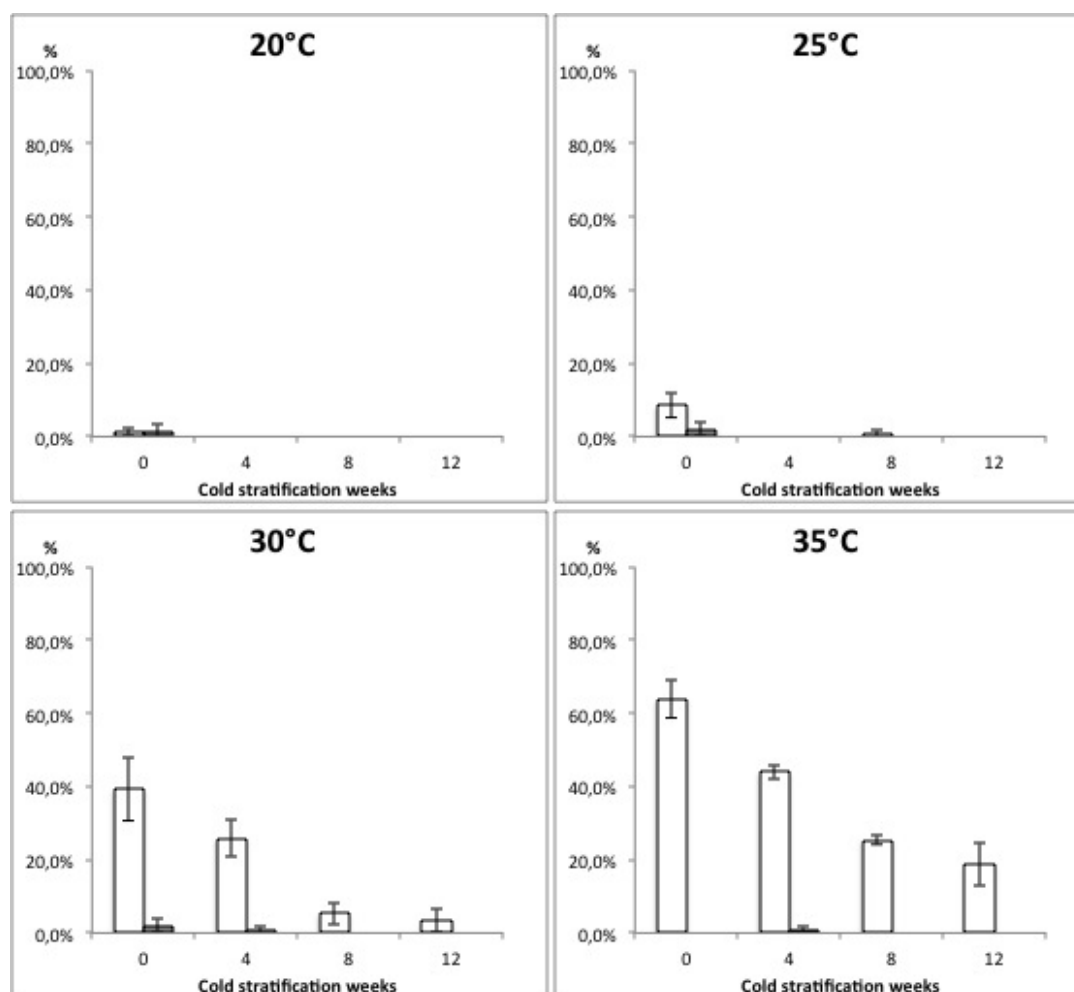


Figure 23 *Chrysanthemum coronarium* var. *concolor* peripheral achenes were stratified at 5° C in darkness for 4, 8 or 12 weeks before imbibition at germination temperatures (20, 25, 30, and 35° C). Fruit coat rupture: open histograms; Seed germination: solid histograms.

Dry storage

Achenes dried for 260d showed the highest germination percentage especially when imbibed at low temperatures, with a maximum germination frequency at 5° C (37,5%), Figure 24.

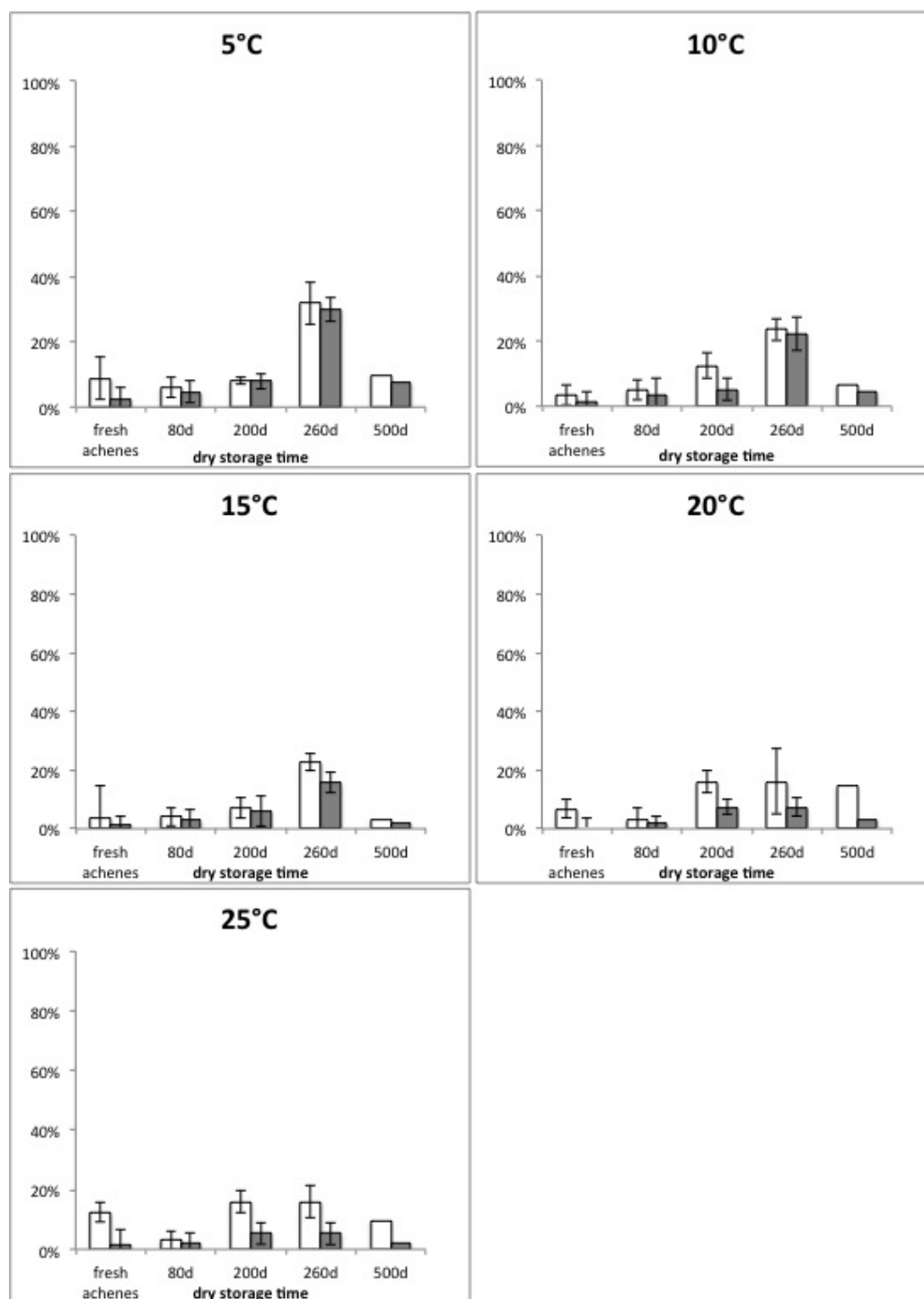


Figure 24 *Chrysanthemum coronarium* var. *concolor* central achenes dry storage for 80d, 200d and 260d. Fruit coat rupture: open histograms; Seed germination: solid histograms.

Dry after ripening

From the dry after-ripening treatment achene germination did not showed significant increase (always lower than 12%) respect to control experiment with fresh achenes.

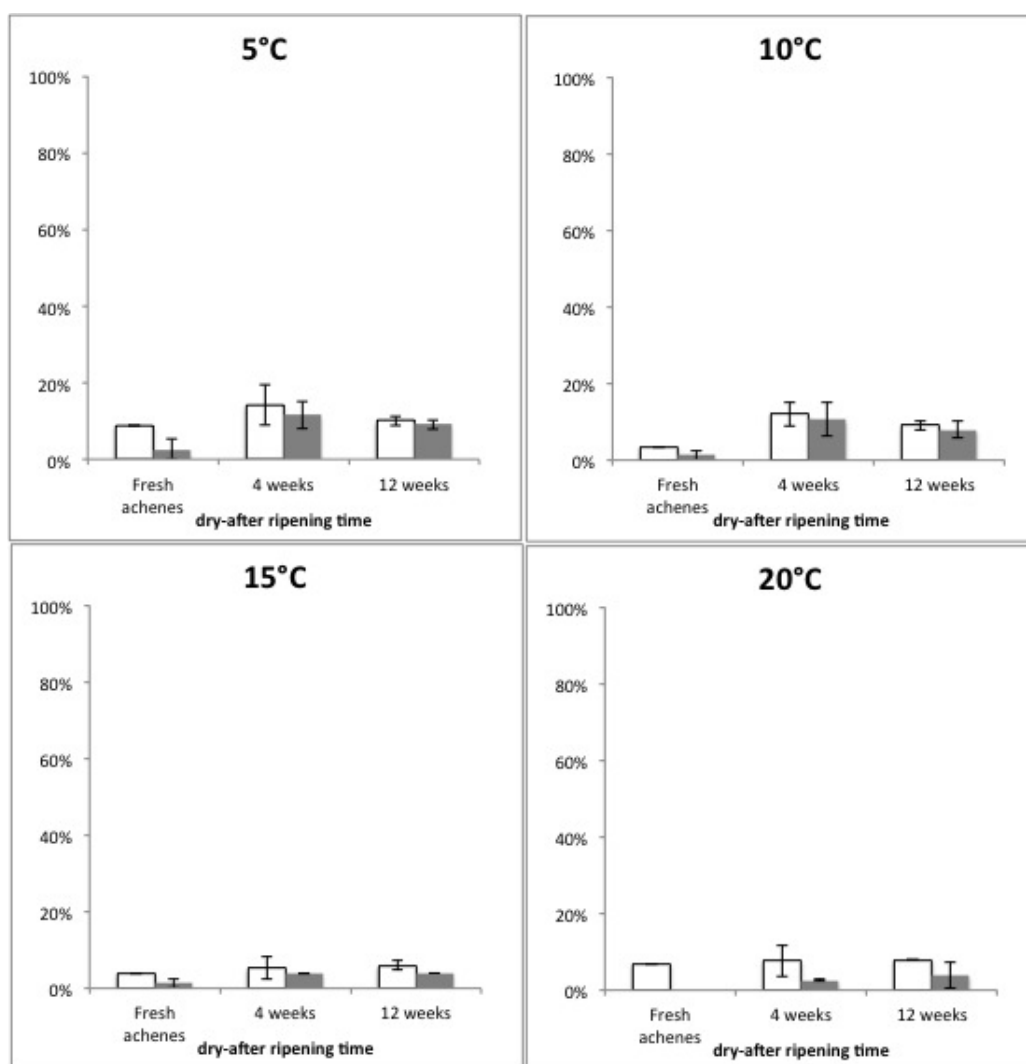


Figure 25 *Chrysanthemum coronarium* var. concolor central achenes dried after-ripening with alternating temperatures of 35/20° C (night/day). Fruit coat rupture: open histograms; Seed germination: solid histograms.

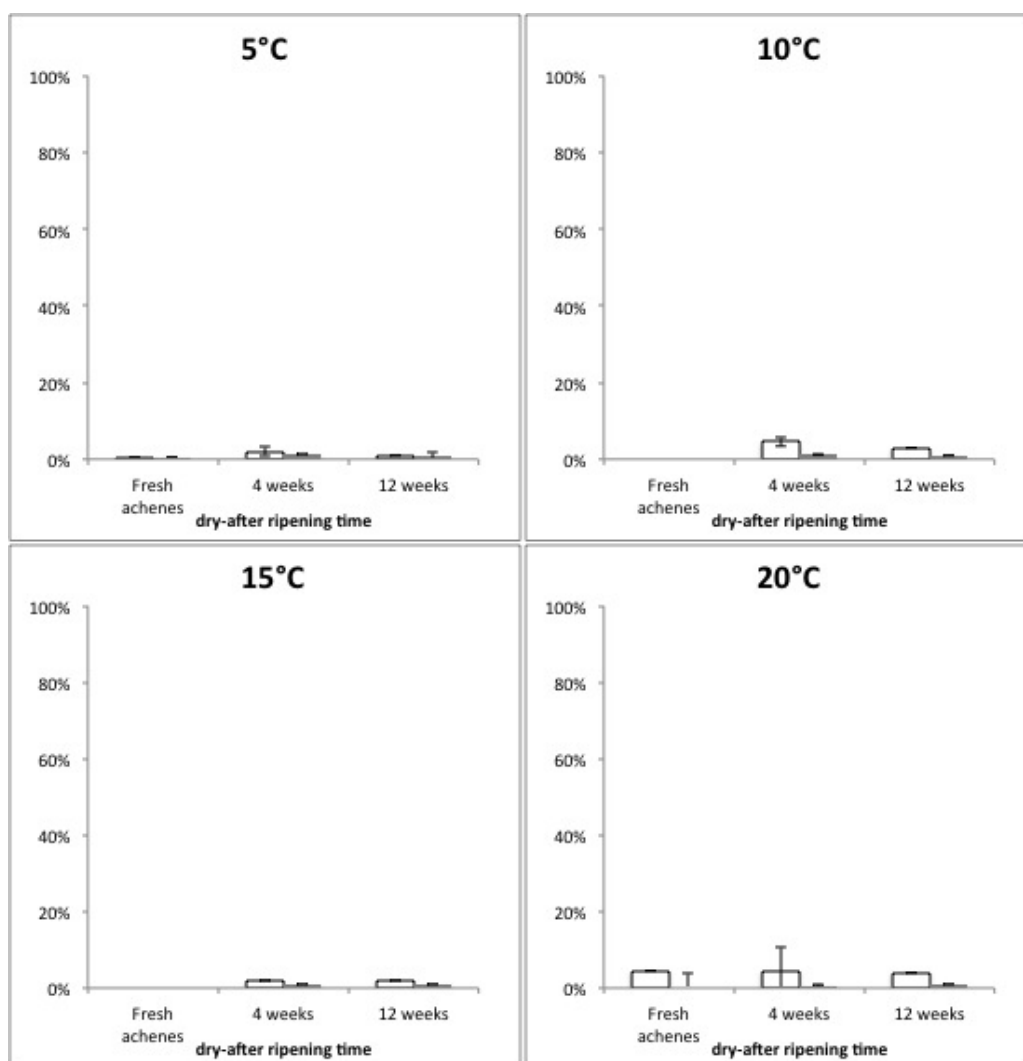


Figure 26 *Chrysanthemum coronarium* var. concolor peripheral achenes dried after-ripening with alternating temperatures of 35/20° C (night/day). Fruit coat rupture: open histograms; Seed germination: solid histograms.

Wet warm stratification

Even though achenes were washed properly with chlorine, during the wet-warm treatment the presence moulds had a detrimental effect in seed surviving. In fact, dead seed percentage reached significant proportions especially in longer-term treatments and always higher in peripheral than in central achenes. Nevertheless, in *concolor* variety, in both morphotypes, wet warm stratification increased achenes germination together with the increasing of time period treatment in central and in peripheral ones. On the other hand, an increase in exposure of achenes to warm for longer time periods meant a much more mould proliferation and seeds death, hence. When achenes were transferred to the germination temperatures (5, 10, 15, and 20° C) lower temperatures (5 and 10° C) resulted in higher germination frequency than higher temperatures (15 and 20° C) and it is probably associated with mould-spread inhibition at lower temperatures. *Discolor* variety behaved similarly showing the same germination pattern (Figure 29 and Figure 30).

Increasing temperature of wet-warm stratification up to 45° C (Figure 31) produced a much faster fruit coat rupture but it caused seed death. Conversely, a wet-warm imbibition at 40° C provided less achenes rupture and a high dead seeds rate (Figure 32). In Table 4 the fruit coat rupture results from drying after ripening and warm-wet stratification treatments are compared.

Treatment ID	Morphotype	Temperature	Water	FCR	Standard Dev.
Drying after ripening for 4 weeks	Central	35°/20°	-	9,7%	0,041
Drying after ripening for 4 weeks	Peripheral	35°/20°	-	3,3%	0,015
Drying after ripening for 12 weeks	Central	35°/20°	-	8,3%	0,017
Drying after ripening for 12 weeks	Peripheral	35°/20°	-	2,5%	0,013
Wet warm after ripening for 3 weeks	Central	35°/20°	+	21,4%	0,016
Wet warm after ripening for 3 weeks	Peripheral	35°/20°	+	30,7%	0,096
Wet warm after ripening for 5 weeks	Central	35°/20°	+	55,4%	0,052
Wet warm after ripening for 5 weeks	Peripheral	35°/20°	+	61,5%	0,028
Wet warm after ripening for 8 weeks	Central	35°/20°	+	72,4%	0,031
Wet warm after ripening for 8 weeks	Peripheral	35°/20°	+	77,8%	0,032

Table 4 Effect of wet-warm treatment and drying after ripening in fruit coat rupture.

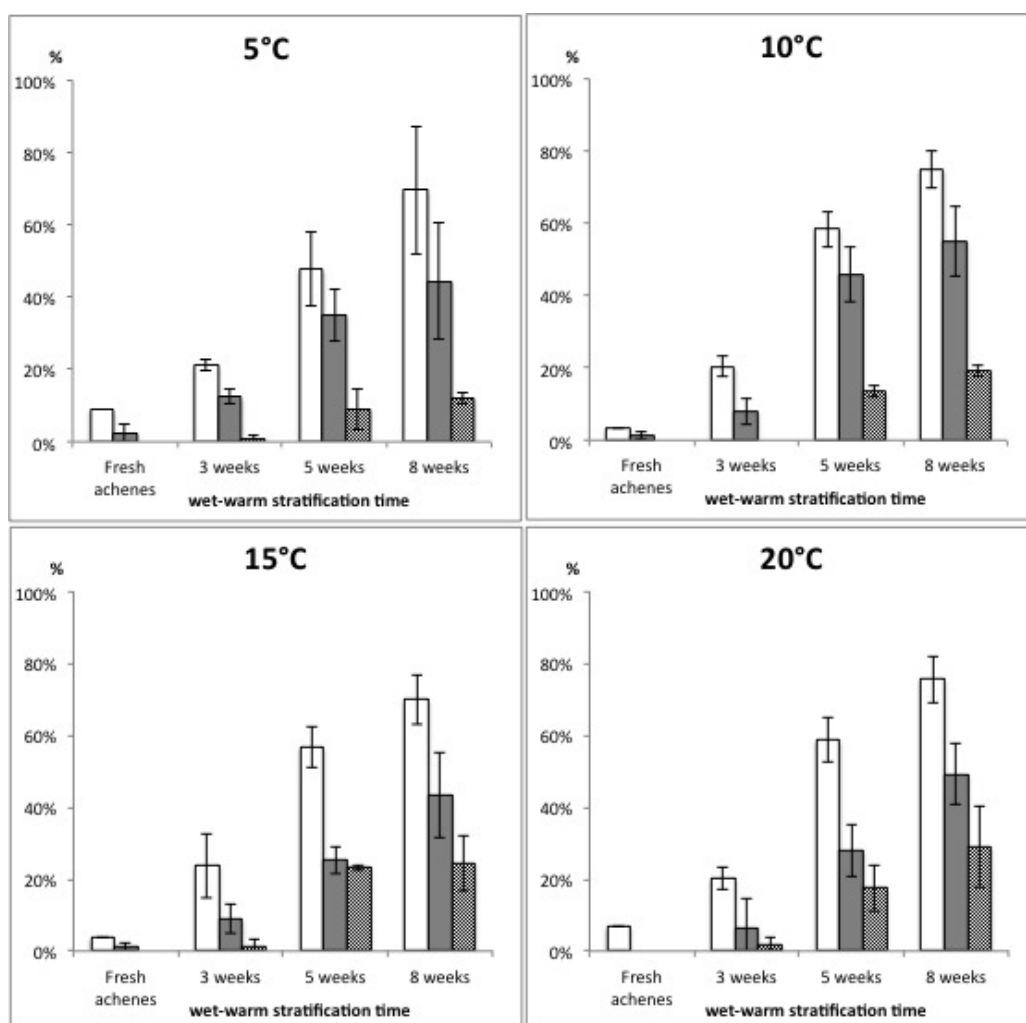


Figure 27 Wet warm stratification after ripening in *C. coronarium* var. *concolor* central achenes. Fruit coat rupture: open histograms; Seed germination: solid histograms; Dead seeds: dotted histograms. In control experiment no seed death was observed.

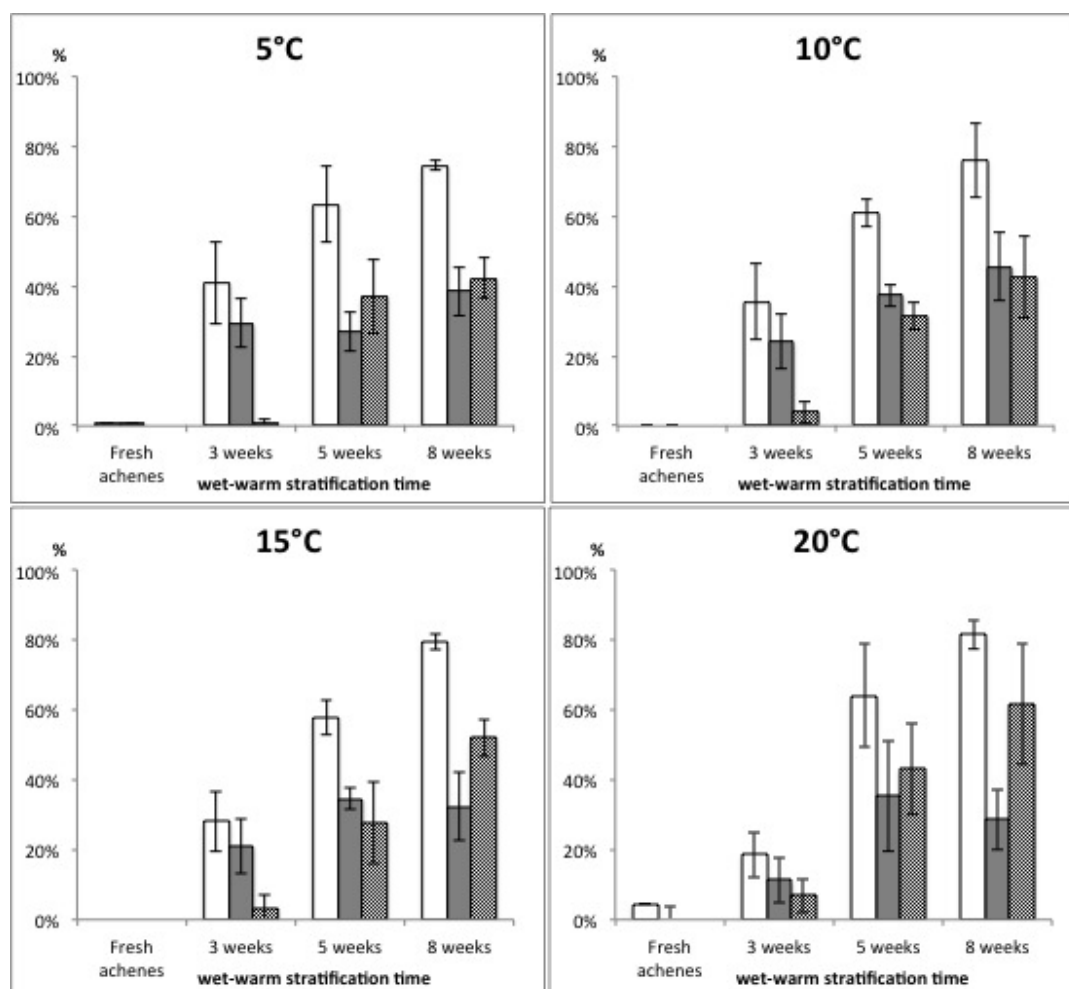


Figure 28. Wet warm stratification after ripening in *C. coronarium* var. *concolor* peripheral achenes. Fruit coat rupture: open histograms; Seed germination: solid histograms; Dead seeds: dotted histograms. In control experiment no seed death was observed.

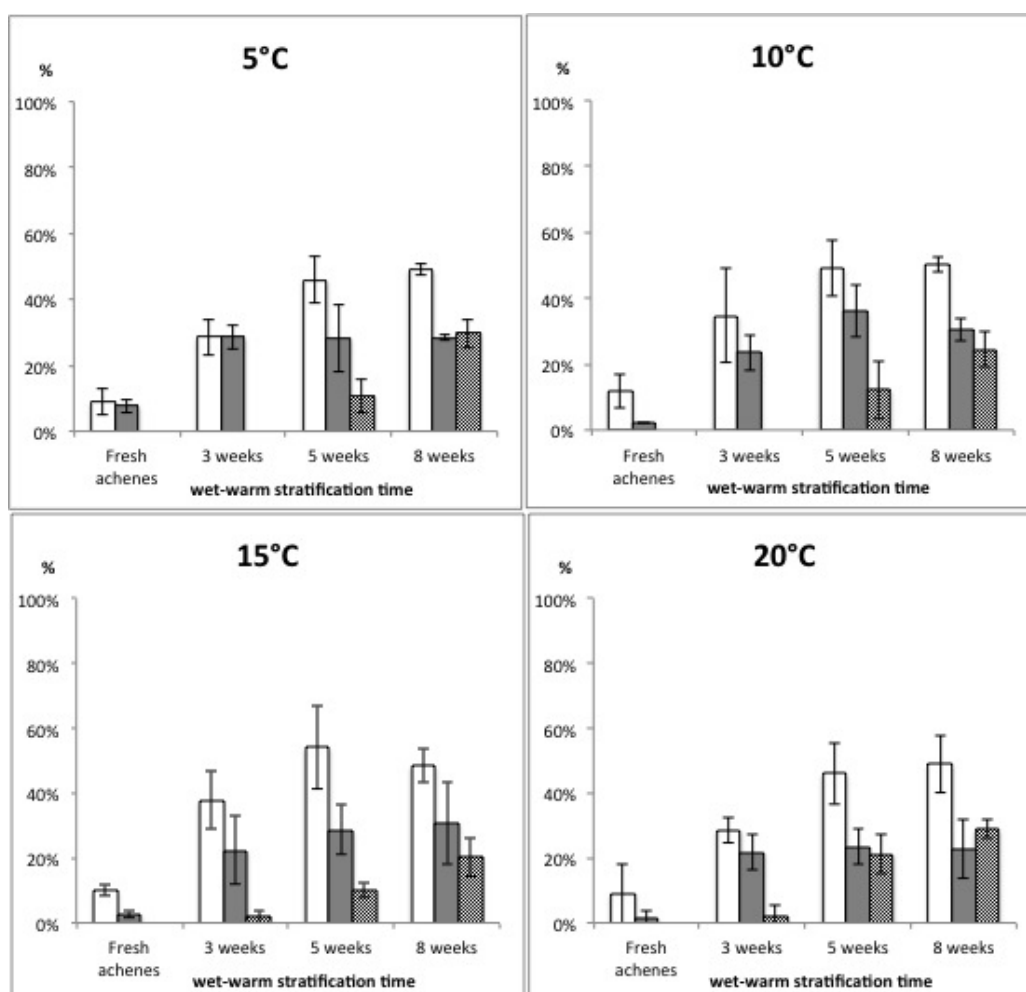


Figure 29 Wet warm stratification after ripening in *C. coronarium* var. *discolor* central achenes. Fruit coat rupture: open histograms; Seed germination: solid histograms; Dead seeds: dotted histograms. In control experiment no seed death was observed.

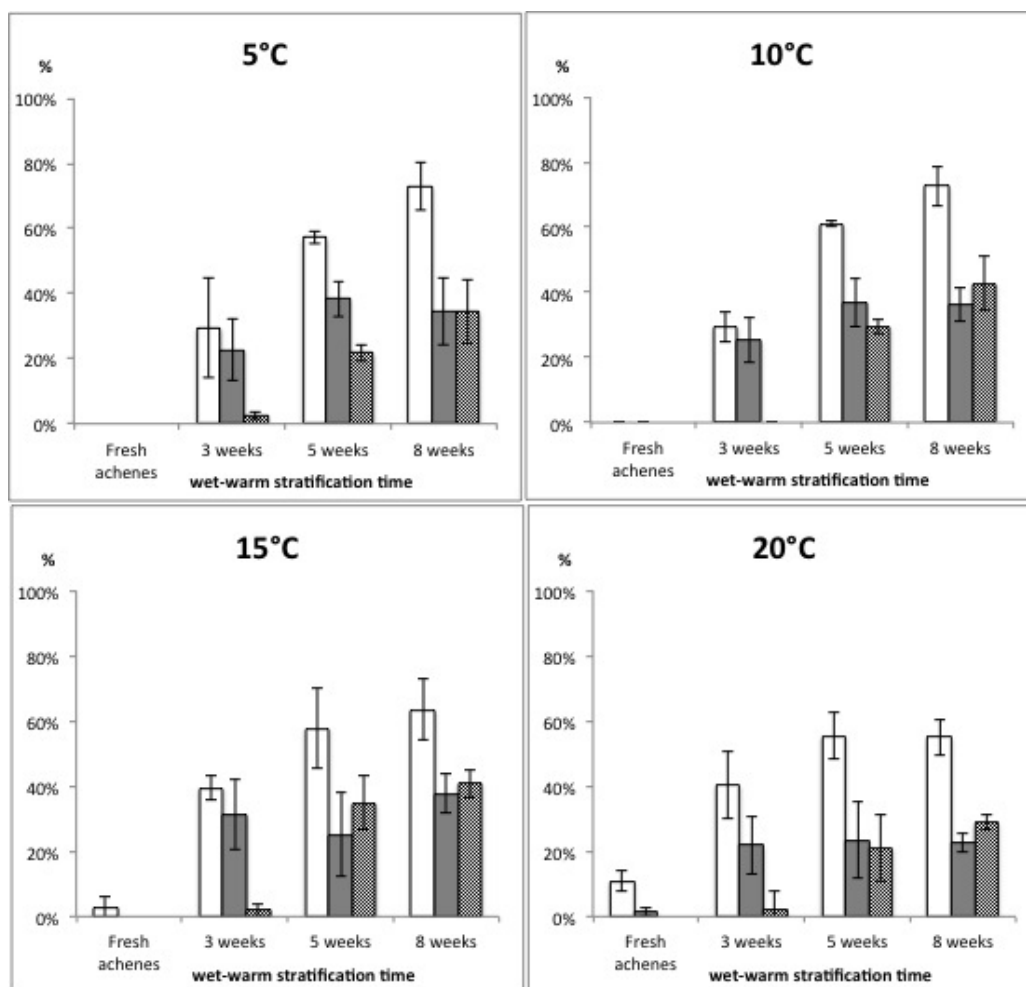


Figure 30 Wet warm stratification after ripening in *C. coronarium* var. *discolor* peripheral achenes. Fruit coat rupture: open histograms; Seed germination: solid histograms; Dead seeds: dotted histograms. In control experiment no seed death was observed.

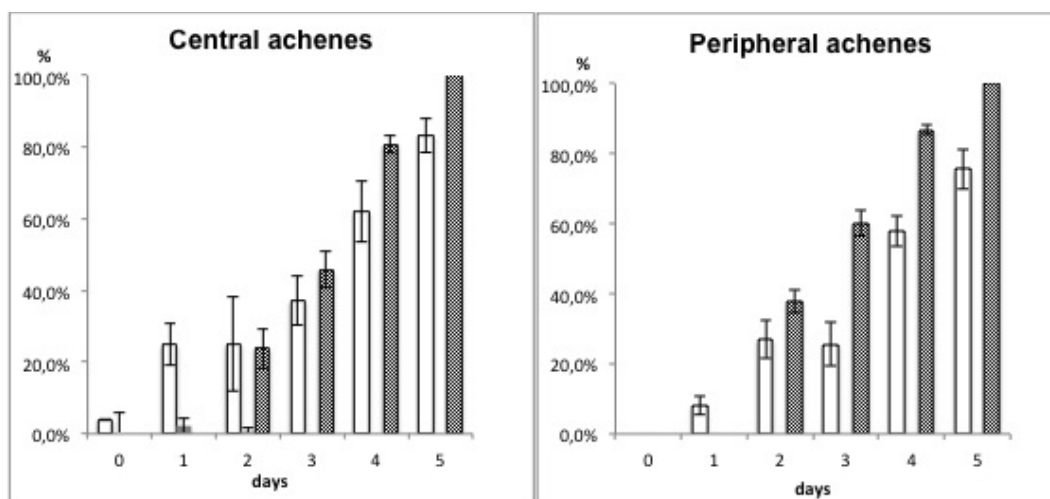


Figure 31 *C. coronarium concolor* wet-warm stratified at 45° C for 1, 2, 3, 4 and 5 days. Fruit coat rupture: open histograms; Seed germination: solid histograms; Dead seeds: dotted histograms.

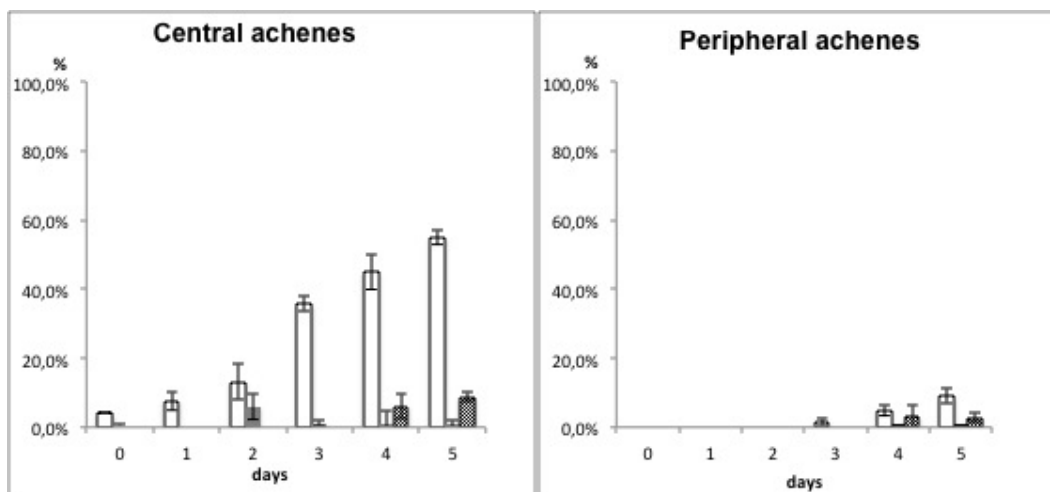


Figure 32 *C. coronarium concolor* wet-warm stratified at 40° C for 1, 2, 3, 4 and 5 days. Fruit coat rupture: open histograms; Seed germination: solid histograms; Dead seeds: dotted histograms.

ABA synthesis

Achenes imbibed at 20° C with different concentrations of fluridone resulted in low germination response (< 5%). The same germination profile was observed when achenes were imbibed with 100uM fluridone after washing for 24h in agitated water.

Genotyping of *Chrysanthemum coronarium concolor* and *discolor* varieties

DNA extraction

An average yield of 3.5 µg of total DNA was obtained from DNA extraction (Figure 33 and Figure 34). Diluted samples resulted in better amplifications (more clear and pronounced bands), but the presence of polyphenol compounds inhibited the *Taq* polymerase activity. Although samples were precipitated and washed again with ethanol the most effective result was obtained by the addition of PVP into PCR mix (Figure 35).

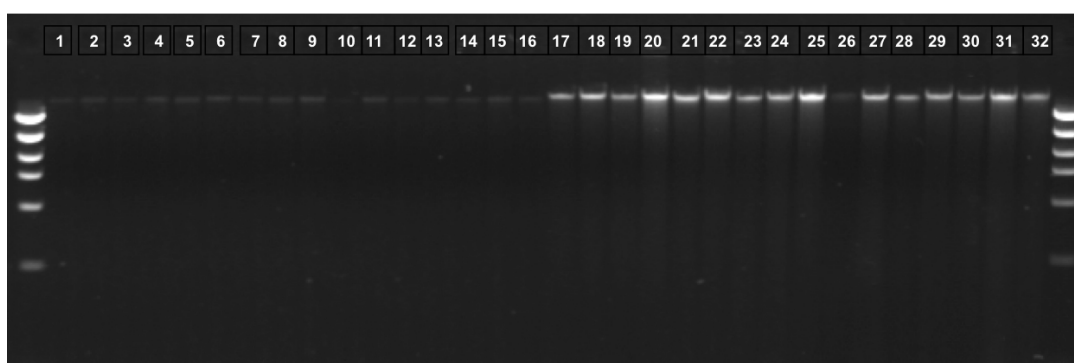


Figure 33 DNA extraction of *Chrysanthemum coronarium* var *concolor*. From 1 to 16 1:10 dilution of extracted samples; from 17 to 32 undiluted samples.

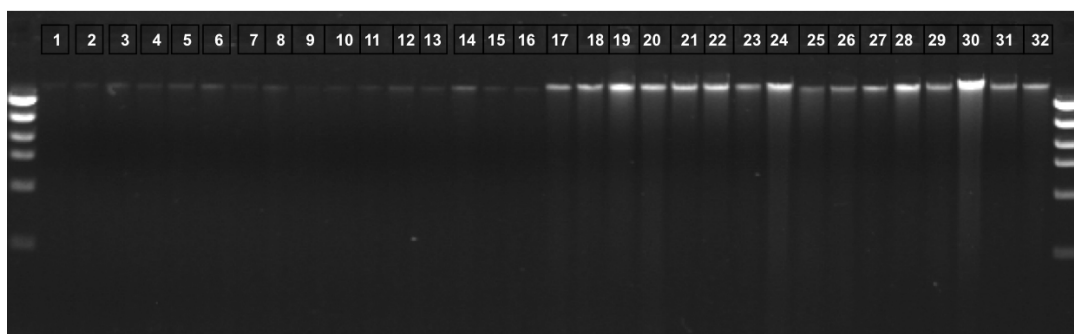


Figure 34 DNA extraction of *Chrysanthemum coronarium* var *discolor*. From 1 to 16 1:10 dilution of extracted samples; from 17 to 32 undiluted samples.

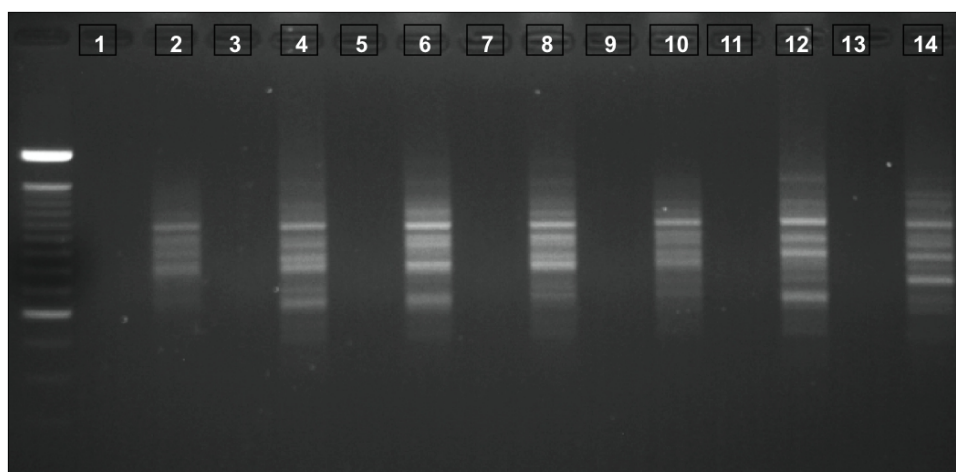


Figure 35 *Chrysanthemum coronarium* var *discolor* amplified with primer Cc_04. 1-2: Cd_10; 3-4: Cd_11; 5-6: Cd_12; 7-8: Cd_13; 9-10: Cd_14; 11-12: Cd_15; 13-14: Cd_16. Odd numbered wells are samples where PVP was not added into PCR mix; Even numbered wells are samples where PVP 2% was added into PCR mix.

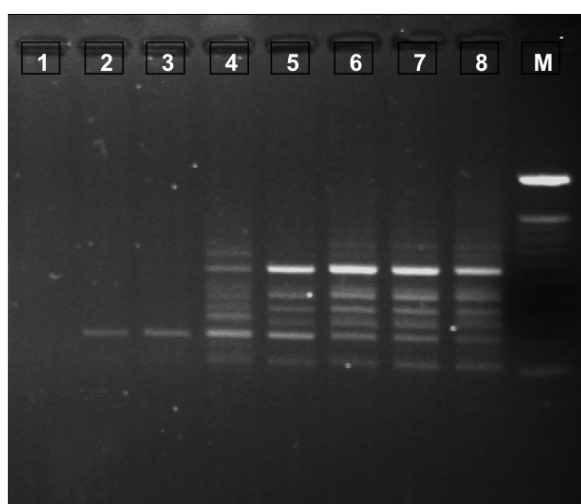


Figure 36 *Chrysanthemum coronarium* var *discolor* amplified with primer Cc_04. Wells 1-4: amplification of Cd_14 sample with, respectively, 0, 1, 1.5, and 2% of PVP in PCR mix; Wells 5-8: amplification of Cd_13 sample with, respectively, 0, 1, 1.5, and 2% of PVP in PCR mix.

Different amounts of PVP in PCR reactions were tested and the addition of 2% PVP produced an increased amount and clearer bands (Figure 36).

ISSR analyses

The final ISSR matrix included 32 individuals and 40 loci, 27 of which were diallelic. We performed a principal co-ordinate analysis (PCoA) of this binary matrix, based on the Euclidean distance, to represent the genetic relationships among populations and individuals (see Figure 37). Because of their capability to represent genetic differentiation, ordination axes obtained from neutral loci matrix can be used to assess correlations between genetic composition and other variables (Lee 2011).

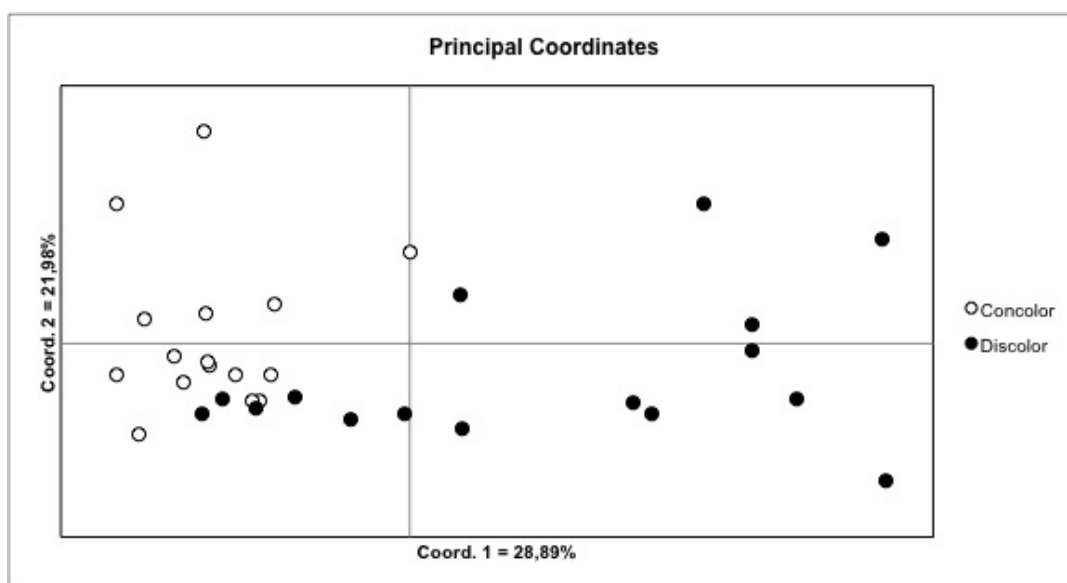


Figure 37 Principal Coordinate Analysis (PCoA) of the two *C. coronarium* varieties.

In PCoA analysis first two axes explain 50,87% of variability. Moreover, assuming the varieties as two distinct populations using PopGene, the genetic identity of the two populations was 0.9443, whereas between the two population was 0.057 (see Table 5)

	Value	st. dev.
Total number of loci	93	
Concolor num polymorphic loci	51	
% of Concolor polymorphic loci	54.84%	
Discolor num polymorphic loci	62	
% of Discolor polymorphic loci	66.67%	
H index	0.222	0.172
I index	0.346	0.242
Genetic identity of the two pops	0.9443	
Genetic between the two pops	0.057	
Ht	0.222	0.029
Hs	0.2	0.0255
Gst	0.1	

Table 5 Genetic variability assessment obtained with ISSR markers.

Sequencing of *rbcL* and *mat-k* regions

The large subunit of ribulose-bisphosphate carboxylase (*rbcL*) gene of 1206 nt was amplified for *concolor* and *discolor* variety of *Chrysanthemum coronarium* (Figure 38). PCR products were directly sequenced using amplification primers.

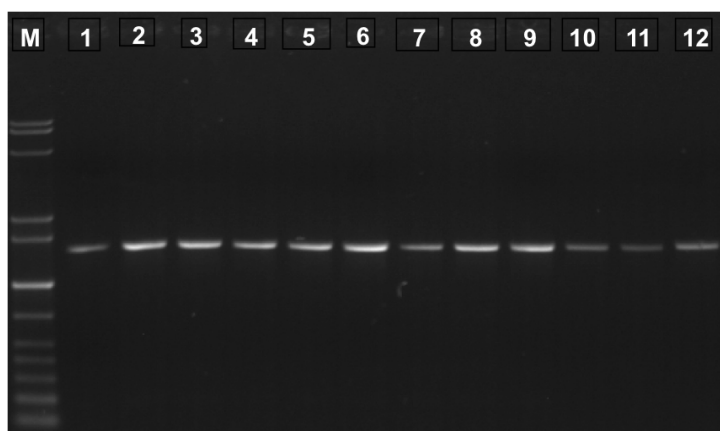


Figure 38 Amplification of *rbc-L* gene in six samples per each variety. 1-6: *C. coronarium concolor* variety; 7-12: *C. coronarium discolor* variety.

Likewise, the plastid region of *mat-k* was amplified (Figure 39).

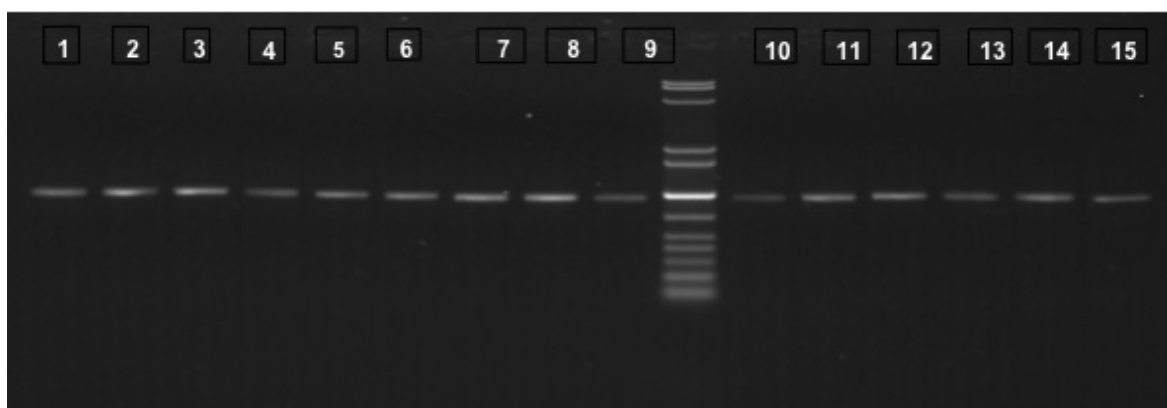


Figure 39 Amplification of *mat-k* gene in seven samples for *C. coronarium concolor* variety (1-7) and eight samples for *C. coronarium discolor* variety (8-15).

The multiple alignment of the *C. coronarium* samples was composed by 2512 sites were only one transversion was observed (see Table 6).

Marker		<i>rbc-L</i>	<i>mat-K</i>
Number of sites		2512	2512
Num of informative sites		0	1
Num of variable sites		0	1
Base composition	% of A	26.9	29.2
	% of C	19.5	18.5
	% of G	24.1	16.2
	% of T	29.4	36.1
Transition		0	0
Transversion		0	1

Table 6 Genetic composition of multiple alignment (*rbc-L* + *mat-k*)

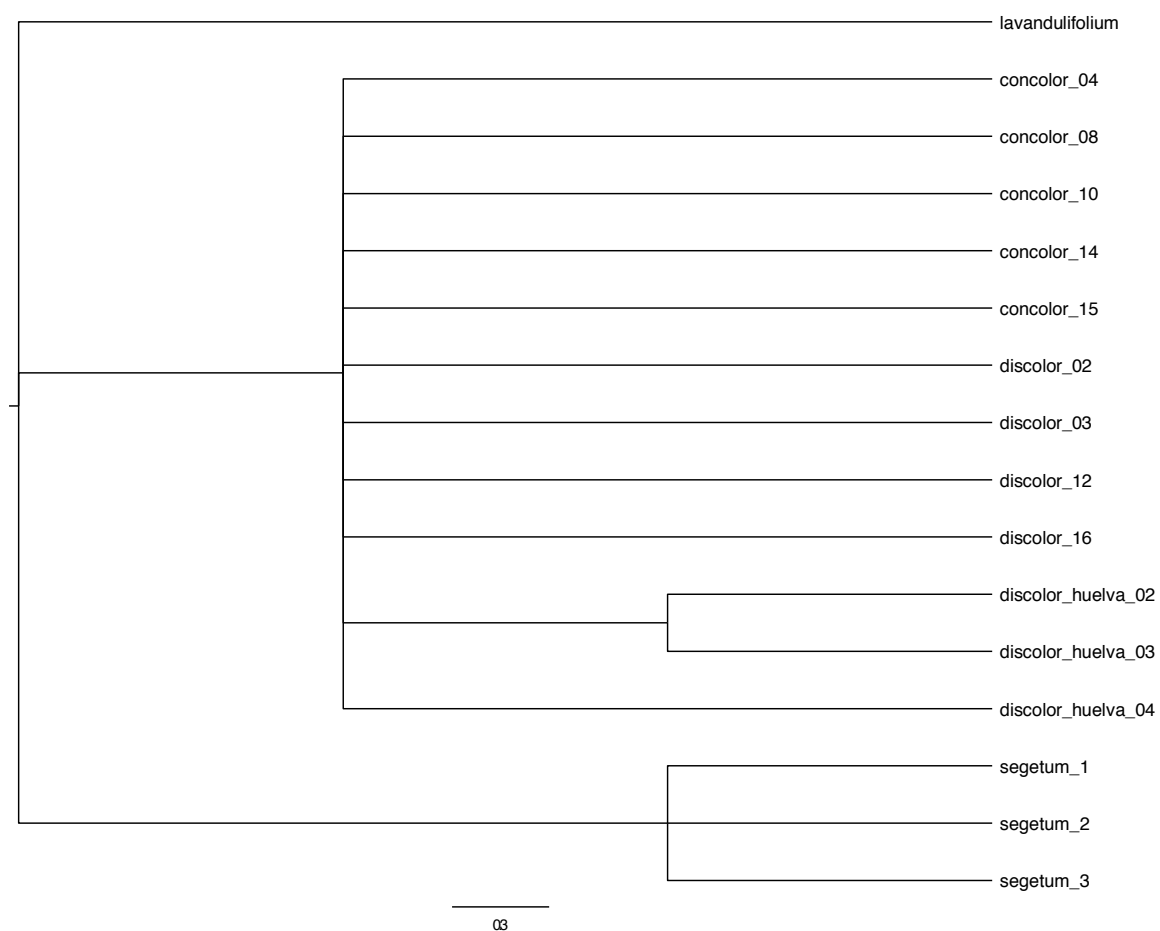


Figure 40 DNA barcode elaborations (rbcL + mat-k). Neighbour Joining tree with bootstrap values reported on the branches.

Three main groups were recognized: 1) *C. lavandulifolium*, 2) *C. segetum* and 3) *C. coronarium* (*concolor* + *discolor*). Within the *C. coronarium* group bootstrap support were not robust enough for varieties differentiation, as among different species.

Physiology of germination in *Leucanthemum vulgare*

Germination behaviour

Leucanthemum vulgare seeds imbibed in water did not showed any dormancy-associated behaviour and germinated in a great amount. In particular, they showed the highest germination percentage when imbibed at 15 - 25° C temperature range (Figure 41) with the probable optimum around 20° C.

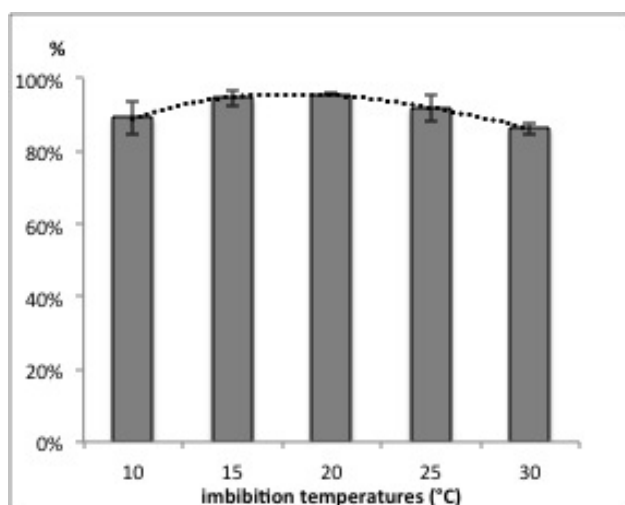


Figure 41 Germination response of *Leucanthemum vulgare* seeds at different imbibition temperatures

Seed osmo-priming

Interestingly, sowing seeds at 10° C after a one-week priming treatment (short-priming treatment) the germination response decreased dramatically (Figure 42). Seeds sowed at higher temperatures were not inhibited by priming, while at 10° C, germination percentage showed a remarkable decrease (-83,2%). Priming seeds for nine weeks (long priming treatment) at 5° C, produced a reversion in germination response and treated seeds behaved as control experiment. Ten-degrees primed seeds, on the other hand, retained priming inhibition in the long-priming treatment as well. Furthermore, nine-weeks priming produced a lower germinability at 30° C, where a significant (26% for 5° C primed seeds and 34% for 10° C primed seeds) of germination drop was observed.

Light during short and long priming treatments produced an increase just in seeds primed at 10° C and imbibed at 10° C where it doubled the germination response in comparison with treatment in darkness (Figure 43).

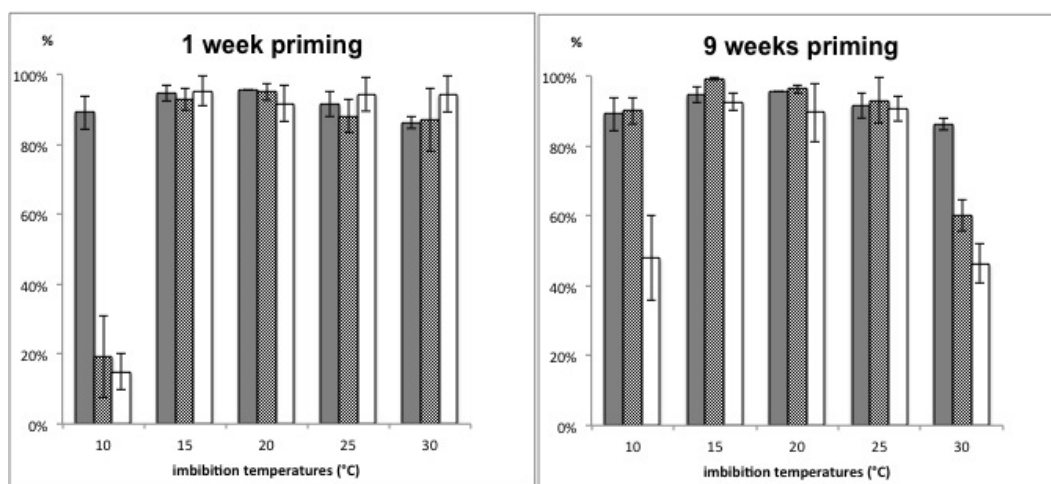


Figure 42 Comparison of the effect of short and long priming treatment on germination behaviour. Solid histograms: imbibition in water (no priming); Dotted histograms: 1 week primed seeds at 5° C; open histograms: 1 week primed seeds at 10° C. In this experiment the priming treatment was carried out in darkness.

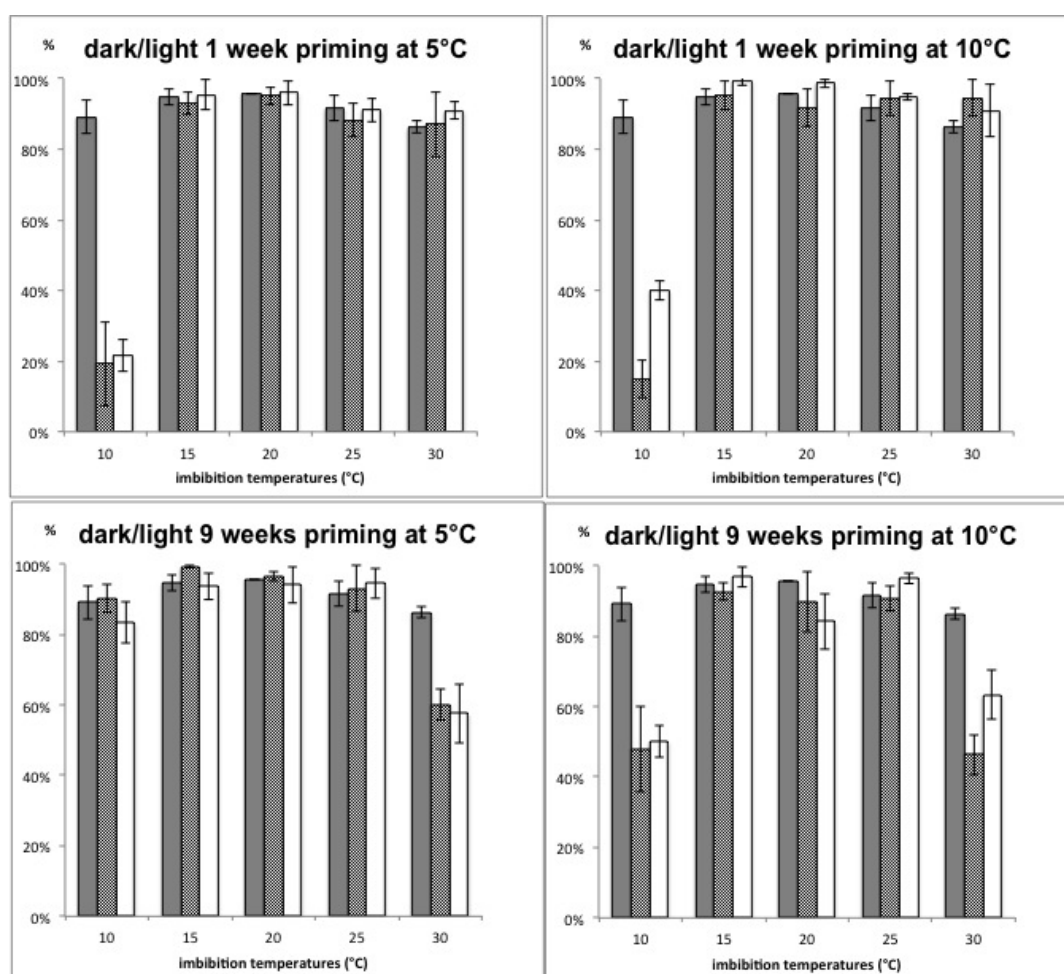


Figure 43 Short and long seed priming with light or darkness. Dotted histograms are dark primed whereas open histograms are primed in light.

Hydro-priming

Sowing seeds at 10° C after a one-week incubation in darkness at 5° C caused a dramatic drop in germination irrespective of osmotic potential of the medium. On the other hand at higher germination temperatures hydro-priming does not produced any remarkable effect (Figure 44).

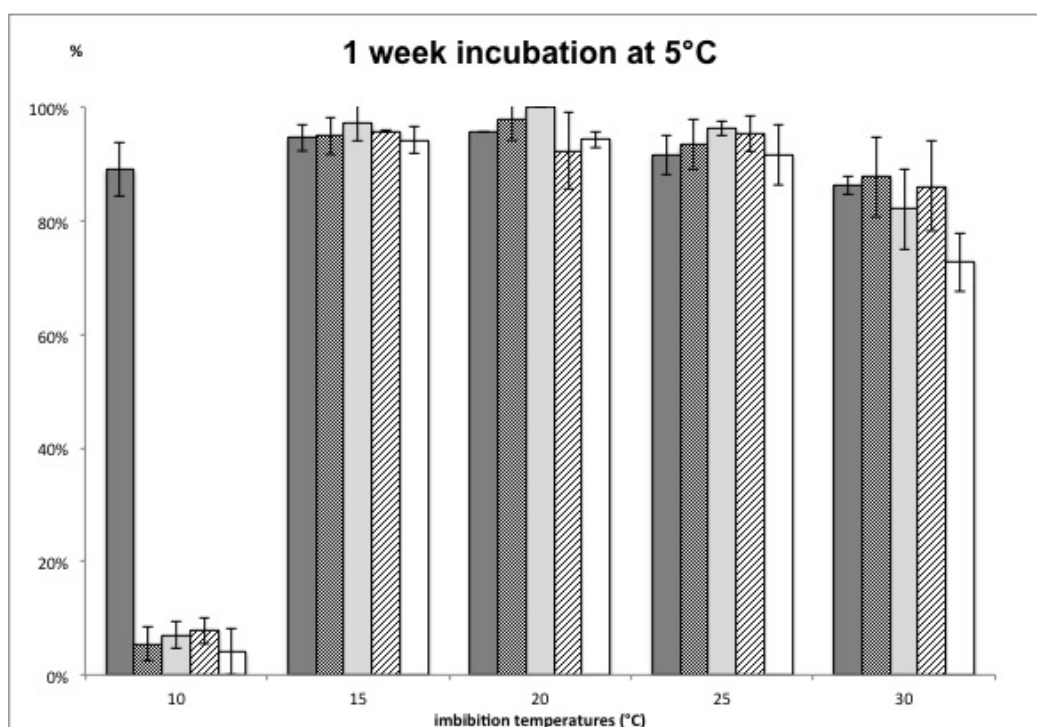


Figure 44 Seed germination of seeds incubated for 1 week at 5° C at different conditions: Solid histograms non-treated seeds; Dotted histograms: seeds imbibed for 1 week in water in dark; Light gray histograms: seeds imbibed in darkness for 1 week with an osmotic potential of $\psi = -0.02$; Striped histograms: seeds imbibed in darkness for 1 week with an osmotic potential of $\psi = -0.072$; Open histograms: seeds imbibed in darkness for 1 week with an osmotic potential of $\psi = -0.292$.

ABA synthesis

Imbibition with fluridone did not produced any significant seed germination increase. Although during osmo-priming seeds were exposed to a low osmotic potential medium and it caused a drop in germination at 10° C, when seeds where sowed with similar osmotic potential conditions (-0.069 respect to -0.072 MPa) seed germination was just partially lowered (from 89 to 60%) (Figure 45).

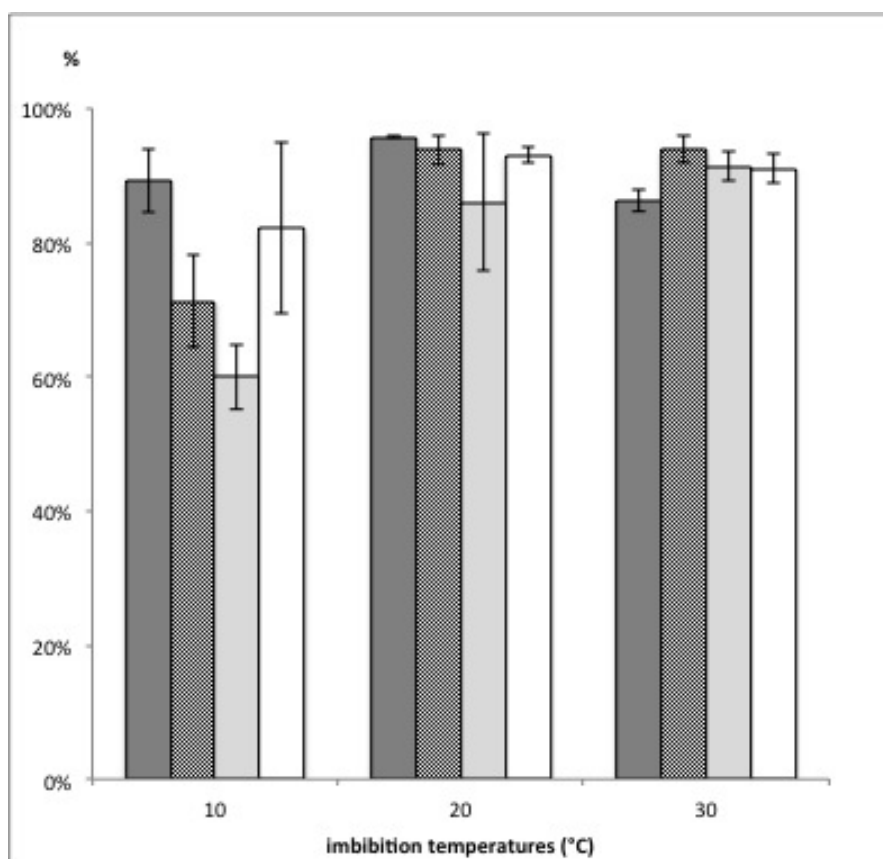


Figure 45 Comparison of germination response among control experiment (solid histograms, imbibition in water), fluridone presence (dotted histograms, imbibition in fluridone 50uM), low osmotic potential medium (light grey histograms, imbibition with a ψ respectively of -0.069, -0.065, and -0.060 MPa) and fluridone together with a low osmotic potential medium (open histograms, imbibition with fluridone 50uM with a ψ respectively of -0.069, -0.065, and -0.060 MPa).

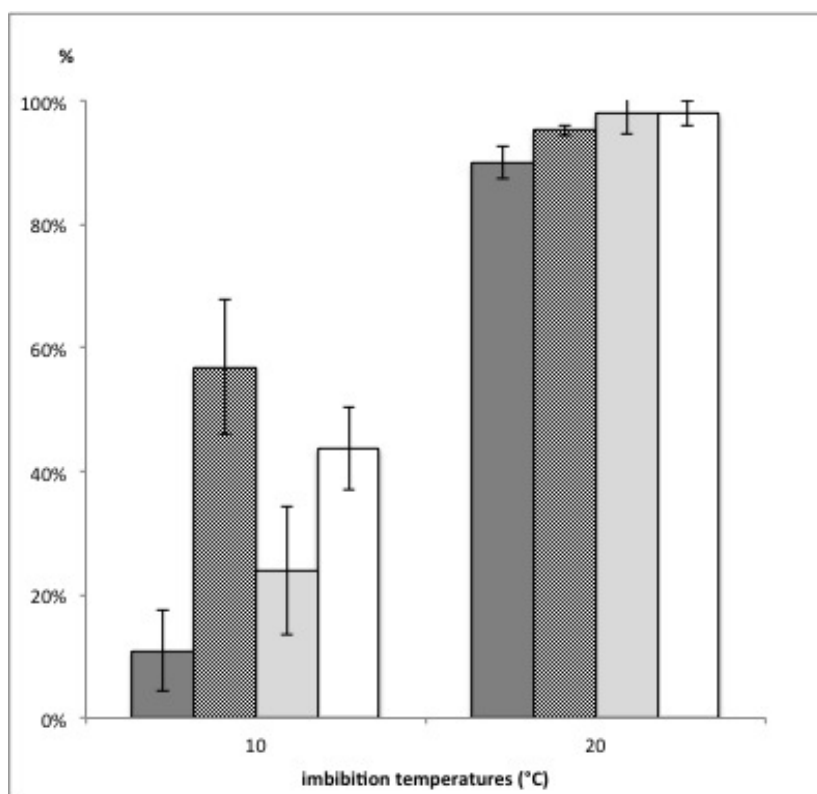


Figure 46 Comparison of germination response among one-week primed seeds at 5° C (solid histograms), one-week primed seeds at 5° C afterwards imbibition with fluridone 50uM (dotted histograms), one-week primed seeds at 5° C with fluridone 50uM afterwards imbibed in a low osmotic potential medium -0.069 MPa (light grey histograms), one-week primed seeds at 5° C with fluridone 50uM afterwards imbibed with fluridone 50uM (open histograms,).

Further experiments with fluridone and gibberellic acid in primed seeds gave significant responses only when one-week primed seeds were imbibed with fluridone 50µM at 10° C (+42%) and with gibberellic acid 1µM at 10° C (+22%) (see Figure 46 and Figure 47).

Analysis of correlation between gibberellic acid/fluridone concentration and germination percentage showed a positive gibberellic acid dose effect ($R^2 = 0,76$) with 5 weeks primed seeds and no correlation for fluridone (Figure 48).

Test	Conditions before germ.	Germination cond.	Germination %	Δ %
Control	-	10° in water	89,2%	-
1w dark	1 week in dark at 10°	10° in water	6,0%	-83,2%
1w PEG at 5°	1 week in PEG at 5°	10° in water	19,0%	-70,2%
1w PEG at 10°	1 week in PEG at 10°	10° in water	15,0%	-74,2%
5w PEG at 10°	5 week in PEG at 10°	10° in water	31,3%	-57,9%
9w PEG at 5°	9 week in PEG at 5°	10° in water	90,0%	+ 0,8%
9w PEG at 10°	9 week in PEG at 10°	10° in water	48,0%	-41,2%

Table 7 Comparison of germination percentage for seeds with different treatments before imbibition

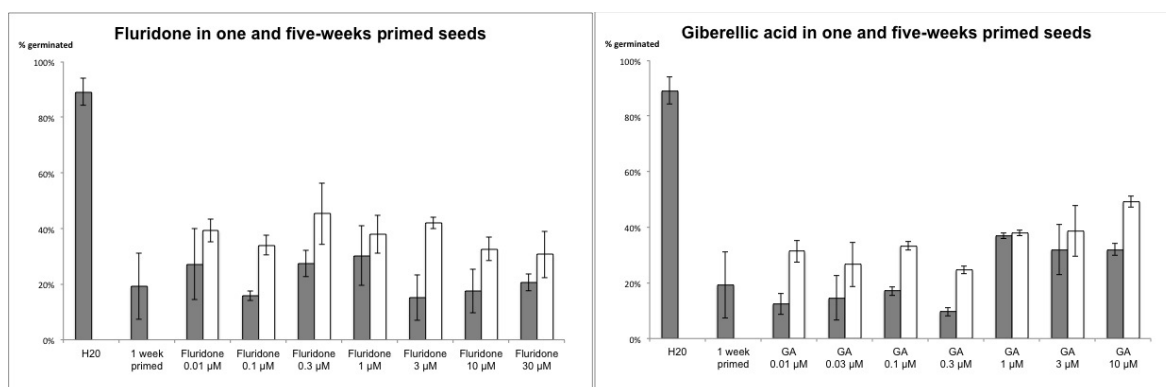


Figure 47 Comparison of different concentrations of fluridone and giberellic acid in one-week primed seeds (solid histograms) and five-weeks primed seeds (open histograms). Final imbibition was carried out at 10° C.

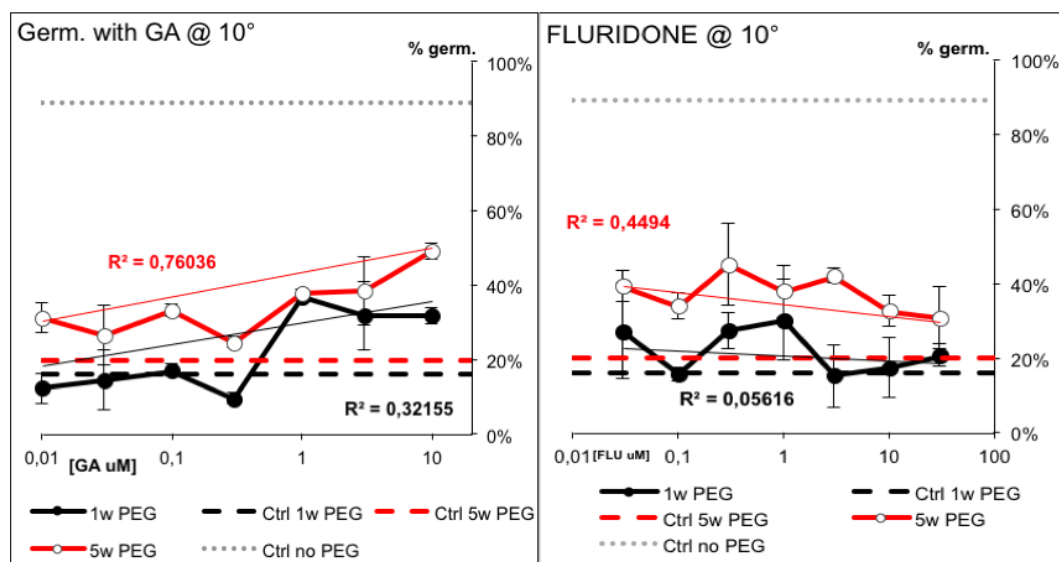


Figure 48 Correlation between giberellic acid/fluridone concentration and germination percentage

Isolations of dormancy genetic markers in *Leucanthemum vulgare* and *Chrysanthemum coronarium*

Genes selection

Five most stable genes were selected as reference genes and their expression level was analysed in a time course graph (Figure 49). Four “Ubiquitin carrier protein” (UBC) genes 8, 12, 21, and 29 together with a “Phospho-glycerate kinase” family protein (PGK) gene, and the 18S ribosomal RNA were selected. From these sequences, Asterids homologous sequence was obtained.

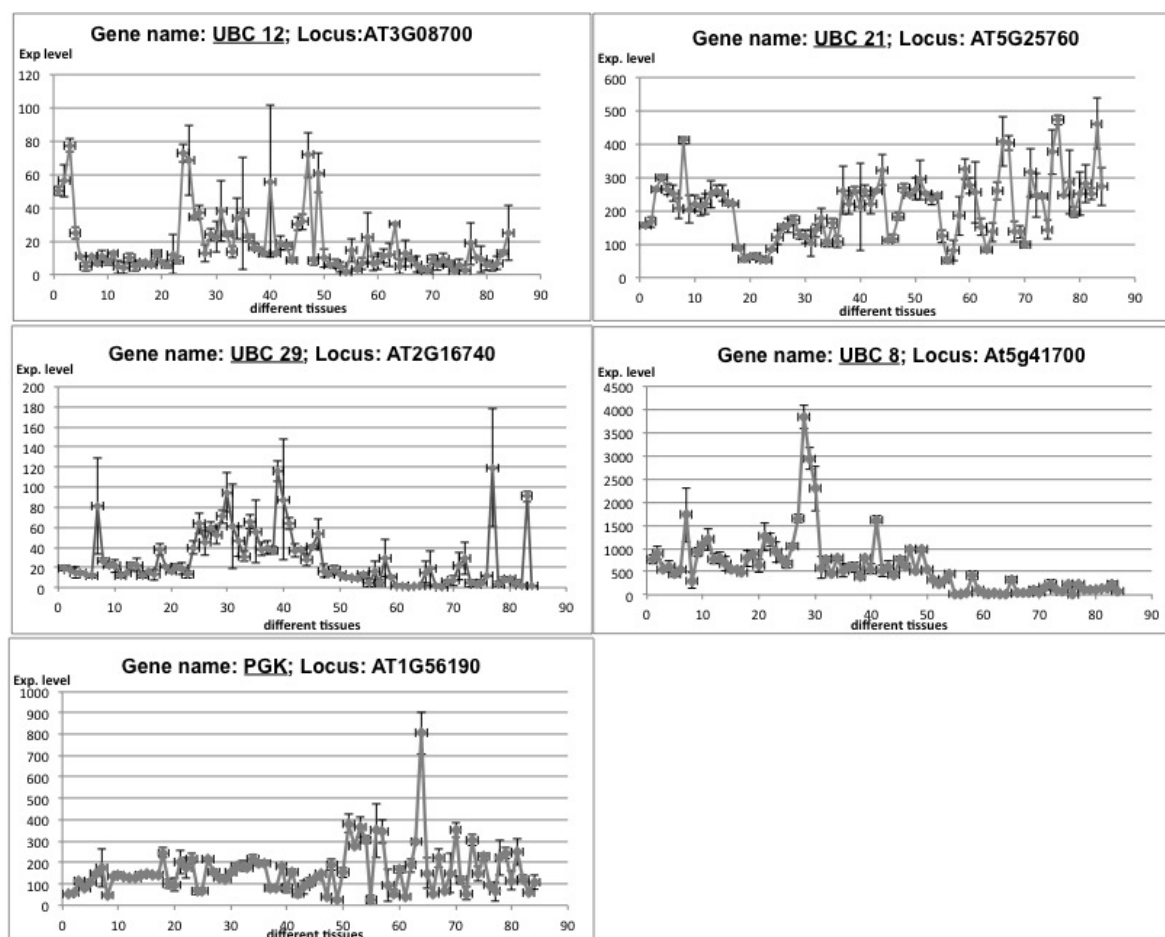


Figure 49. Expression level analysis of reference genes in different tissue and developmental stage in *Arabidopsis thaliana* (rough data from <http://www.arabidopsis.org/> web portal).

For dormancy-associated genes, *DOG1*, *FLC*, and *HUB2* Asterids homologous sequences were obtained.

Starting from homologous sequences alignments 62 new cloning were designed and 42 (Table 8) of which were used for PCR amplifications.

Oligo ID	3'-5' Primer sequence	Description	Use in this study
UBC_9	GATTTTGAAGGAATTGAAGG	ubiquitin-conjugating enzyme	reference gene
	TACTTCTGTGTCCAGCTACG		
UBC_10	TGGCTTCGAAAAGGATTTTG	ubiquitin-conjugating enzyme	reference gene
	TGGCGTACTTCTGTGTCCAG		
UBC_8	ATGGCTTCAAAGCGGATCTT	ubiquitin-conjugating enzyme	reference gene
	CAGCTGCGTGCAGTAGTTTC		
UBC_28	GAAAGAGCTCAAGGATCTTC	ubiquitin-conjugating enzyme	reference gene
	CCATGGCATACTTTTGG		
	CAAAGCGGATCTTGAAA		
	GCATACTTTTGGGTCCAG		
UBC_21	AGGCATCAAGAGCAAGACT	ubiquitin-conjugating enzyme	reference gene
	CTGCAAGCTTGGTATACATC		
	TGCAGGCATCAAGAGC		
	AGCCATAGACTGAAATCCTC		
PGK	GAAGAACGATCCAGGCTTTG	phosphoglycerate kinase	reference gene
	CACCCATCTGGGATAGCAGT		
	CTTCCTGATGGTGGTGTT		
	ATCTGGGATAGCAGTGGA		
18s RNA	CTCAAAGATTAAGCCATGC	ribosomal component	reference gene
	TACGAGCTTTTAACTGCAA		
	GAGCTAATACGTGCAACAAA		
	CCTTACTATGTCTGGACCTG		
DOG1_f1	CTAAGGAAAAATGGTGAACAA	Delay of Germination	Dormancy-associated gene
	TCTCTTTGTTGCTGCTAAGT		
	ACGACGAATTGAATGAGTT		
	ATATGCCCAATATCTCCTTT		
	TTGAATGAGTTACTAAG		
	TCTTCTAGTAAATTTGACAT		
FLC_f1	ATCGATATGGGAAACA	Flowering Locus C	Dormancy-associated gene
	AWCGATATSGSAAMCA		
	GCTCTTCTCGTCGTCTC		
	GTCTTCTTGGYTCTRRT		
Hub2_f1	CTCCAGCATCAAAATCAGA	Histone H2B Monoubiquitination	Dormancy-associated gene
	TTCAGCTGAGCTTCCATCAT		
	AAAATGCAGAAAGATGTGGCA		
	ATGATGGAAGCTCAGCTGAA		
	ATTACAACCTCTTTTGCC		
	ATGATGGAARCWCAGYTRAA		
	ATTACMACCTCYTTKGGYC		
EXP	ACGACCCTAAATGGTGCTTG	Expansin	Dormancy-associated gene
	TTGTGATGGTTCTTCCGTCA		

Table 8 Cloning primers list of dormancy-associated genes and reference genes

DNA extraction

Four different replicas were obtained from DNA extraction and a 1:1000 dilution was used as template for gDNA quality control with Expansin primers designed for *Arabidopsis thaliana* in PCR (Figure 50).

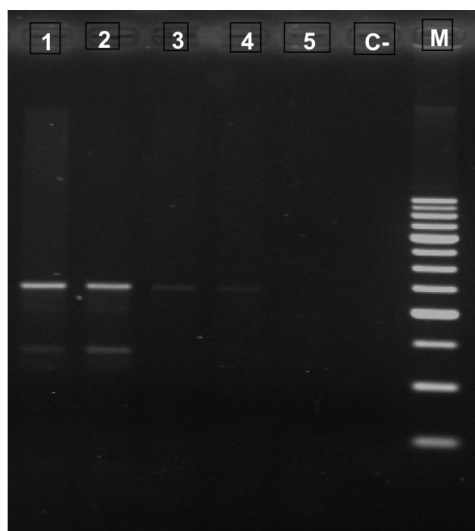


Figure 50 PCR amplification of extracted gDNA. 1 = *L. vulgare* replica 1 (with testa tissue); 2 = *L. vulgare* replica 2 (with testa tissue); 3 = *L. vulgare* 1:1000 dilution template 1 (without testa tissue); 4 = *L. vulgare* 1:1000 dilution template 2 (without testa tissue); 5 = *Succisa pratensis* DNA extraction. C- = PCR negative control; M = marker.

RNA extraction

Extracted RNAs from various treated tissues were quantified and analysed through electrophoresis runs (Figure 51 and Figure 52).

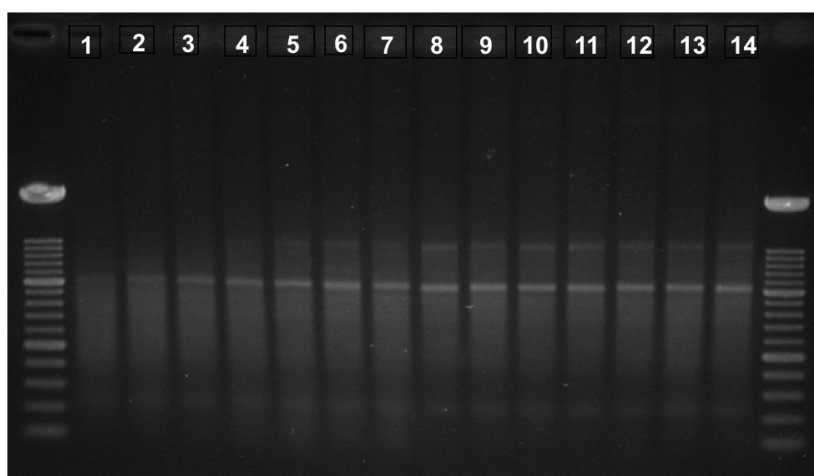


Figure 51. Wells 1-7 = five weeks After drying replicas; 8-10 = 24 hours after imbibition at 30° C replicas; 11-13 = 24 hours after imbibition at 20° C replicas; 14 = 24 hours after imbibition at 10° C replica.

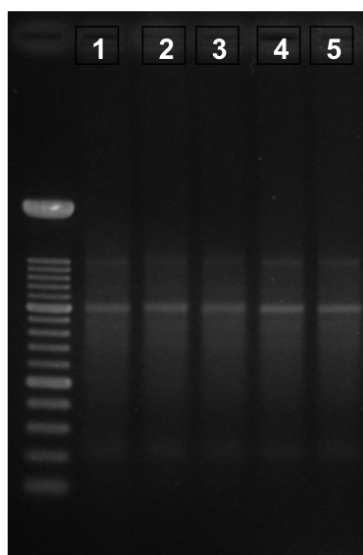


Figure 52. Wells: 1-2 = 24 hours after imbibition at 10° C replicas; 3-5 = 24 hours after imbibition at 20° C replicas.

From the obtained RNAs samples cDNA first synthesis was carried out and checked in a control PCR using *A. thaliana* Expansin primers (Figure 53).



Figure 53. Expansin amplification with cDNA as template

PCR products cloning with TA-Vector

From the gDNA and cDNA of *L. vulgare* and with *C. coronarium* gDNA, reference and dormancy associated genes were amplified. Amplified samples were selected basing on the band analysis (especially taking into consideration the size of the predominant band size compared to the supposed sequence length). Twenty-one PCR products samples from

Chrysanthemum coronarium gDNA amplifications were chosen for plasmid cloning (Figure 54).

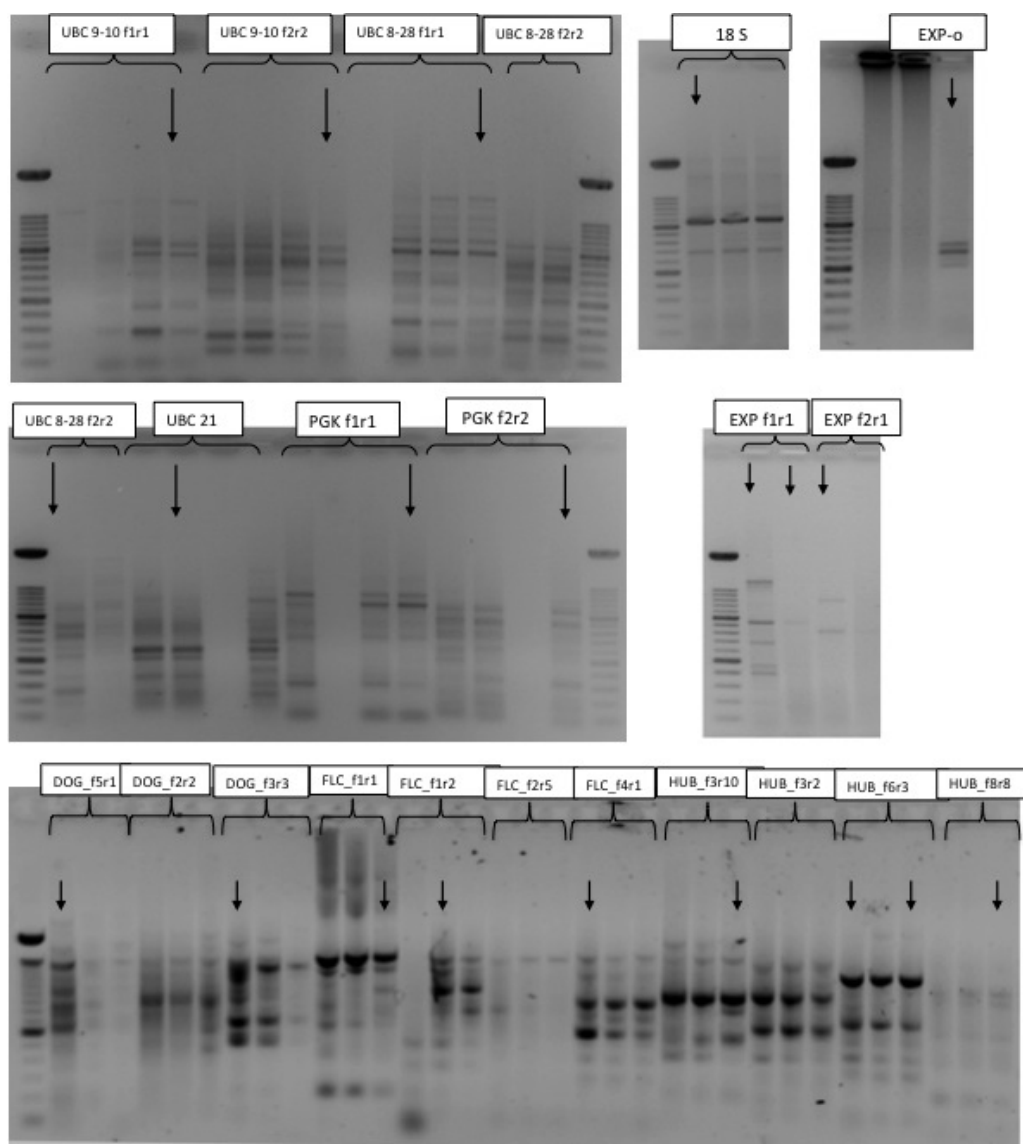


Figure 54 Amplification of reference and dormancy-associated genes using gDNA of *Chrysanthemum coronarium* as template. Arrows indicate the PCR product selected for plasmid cloning.

Likewise, from the amplifications with *Leucanthemum vulgare* twenty-four PCR products samples using gDNA as template (Figure 55), and eight PCR products using cDNA as template (Figure 56) were used for plasmid cloning.

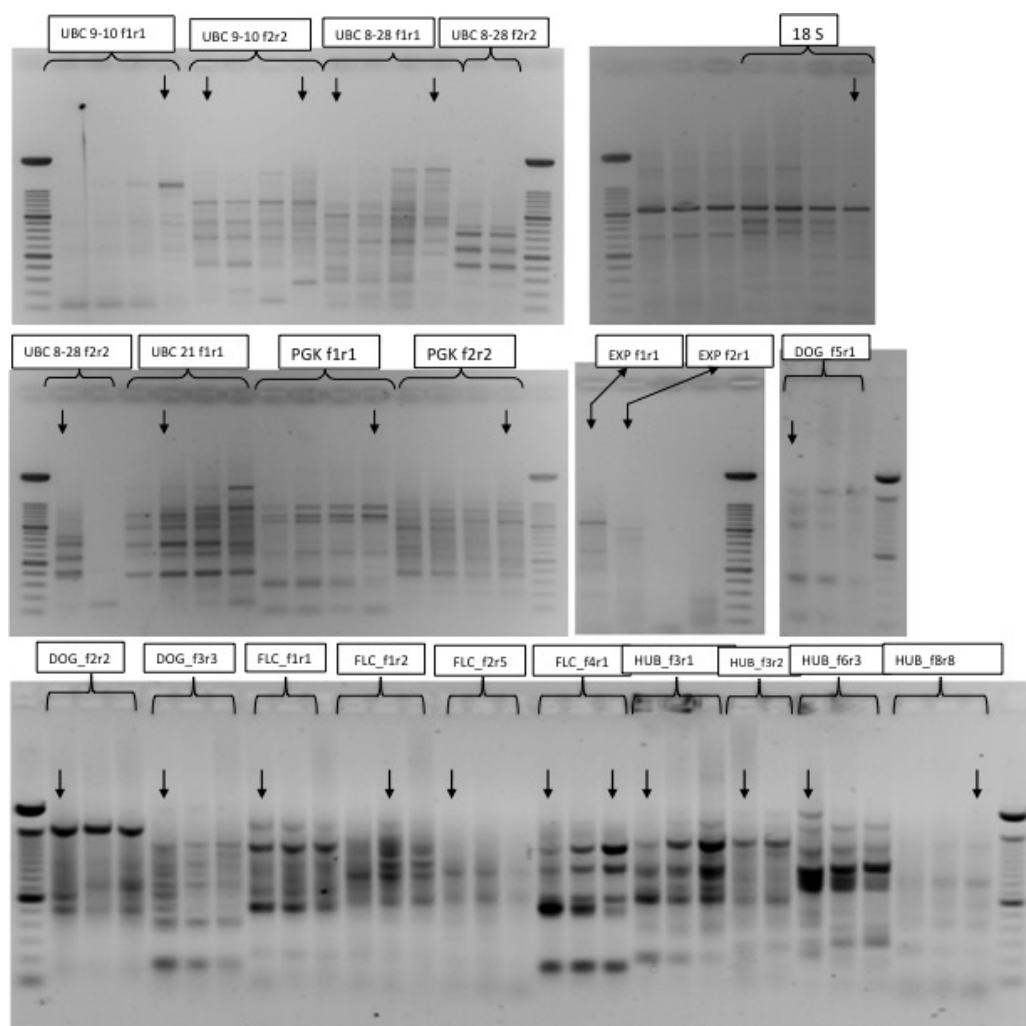


Figure 55 Amplification of reference and dormancy-associated genes using gDNA of *Leucanthemum vulgare* as template. Arrows indicate the PCR product selected for plasmid cloning.

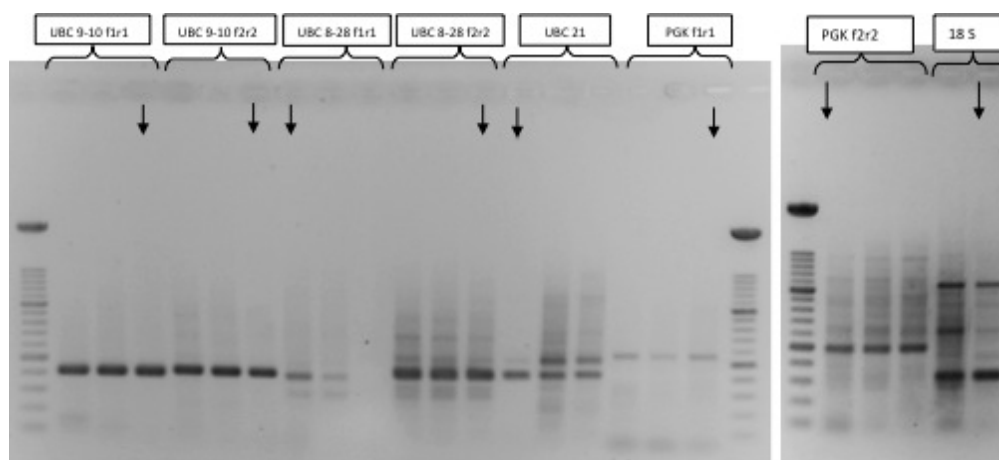


Figure 56 Amplification of reference and dormancy-associated genes using cDNA of *Leucanthemum vulgare* as template. Arrows indicate the PCR product selected for plasmid cloning.

From the PCR colony screening (see Figure 57, Figure 58 and Figure 59 as examples of the colony PCRs carried out), 119 clones were identified for DNA sequencing and 84 of which gave suitable sequence reads.

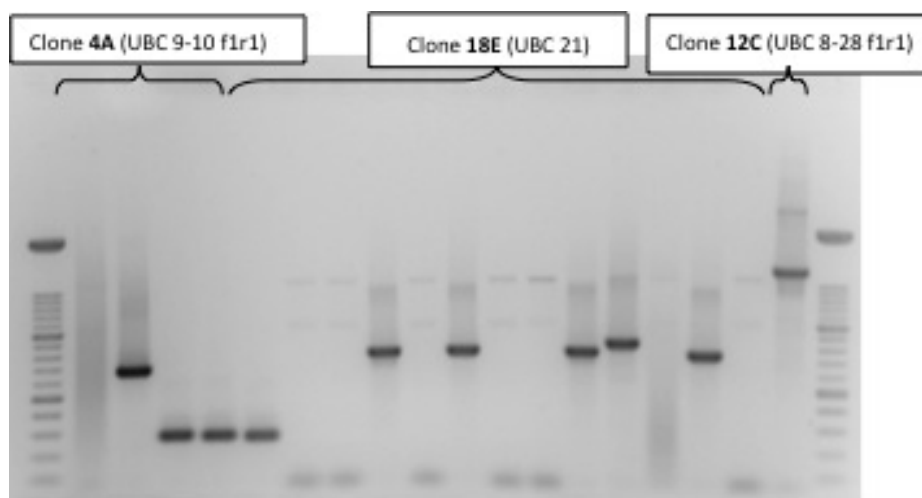


Figure 57 Some of the colony PCRs carried out starting from *Chrysanthemum coronarium* gDNA as template. Colonies with longer inserts were used for DNA sequencing.

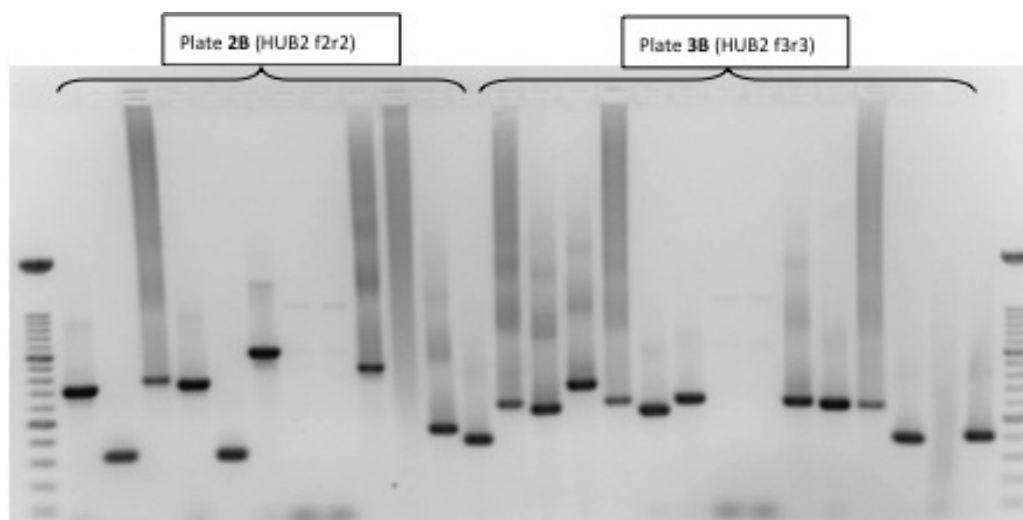


Figure 58 Some of the colony PCRs carried out starting from *Leucanthemum vulgare* gDNA as template. Colonies with longer inserts were used for DNA sequencing.

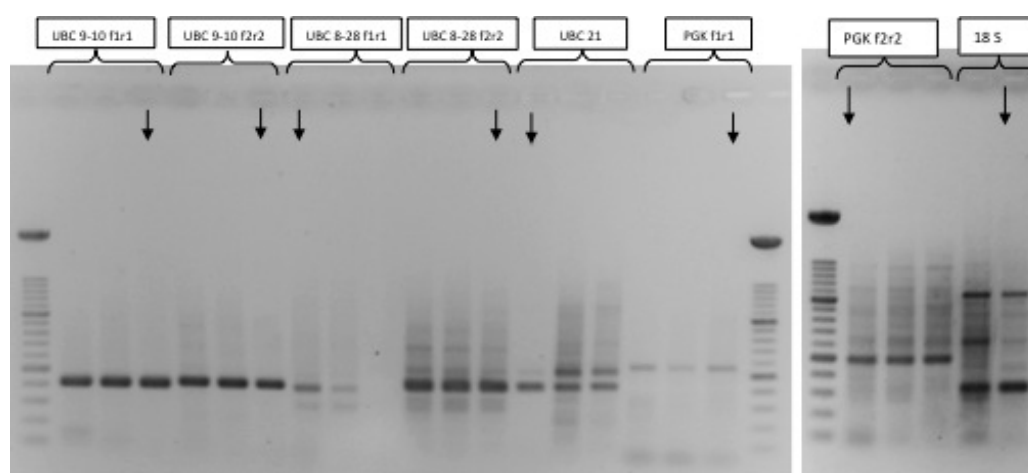


Figure 59 Some of the colony PCRs carried out starting from *Leucanthemum vulgare* cDNA as template. Colonies with longer inserts were used for DNA sequencing.

Sequences analyses

Phosphoglycerate kinase (PGK) gene

Using *C. coronarium* chromosomal DNA as template significant results in BLAST search were obtained. In particular “PT_28-13” sequence provided a specific hit with *PGK* gene. In the alignment with homologous sequences *C. coronarium* clearly clustered with *Helianthus* (Figure 60).

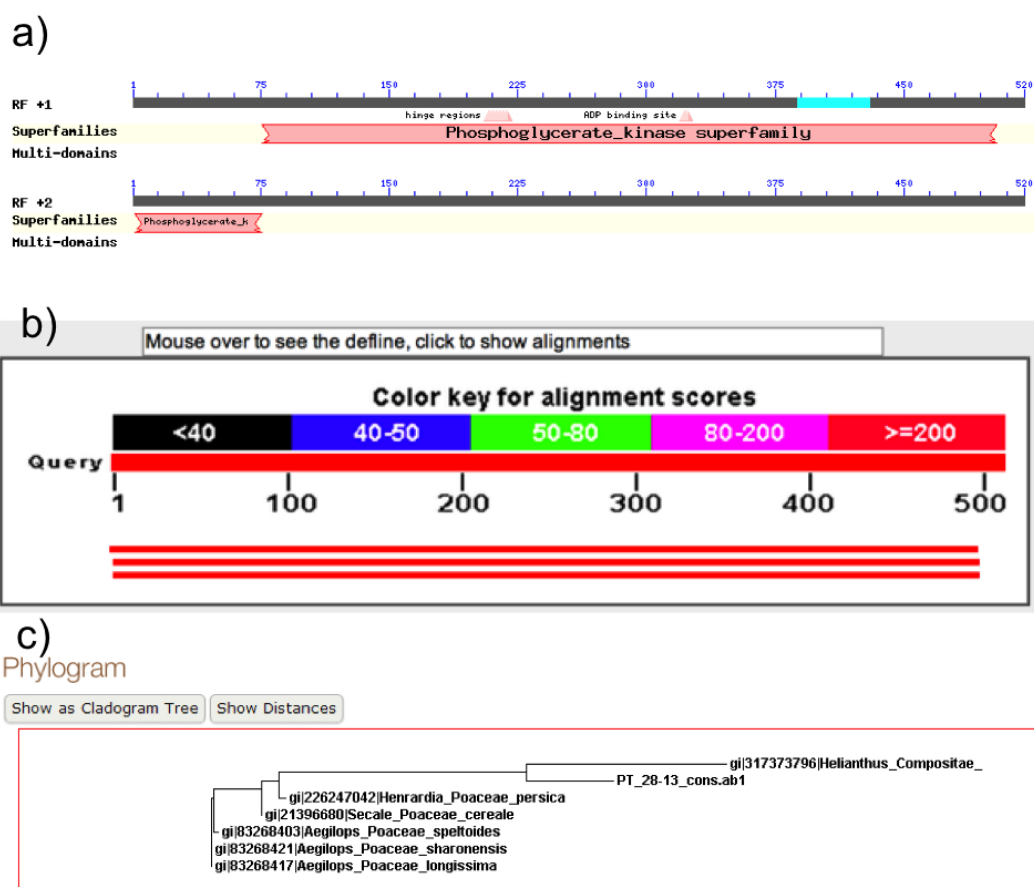


Figure 60 a) Protein domain, BLASTx search; b) Nucleotide BLASTn search; c) Phylogram obtained from sequences alignment.

18s gene:

Both species provided similar results with a high hit score specific for *18S* gene. Alignment with *Helianthus* 18S rDNA sequence showed that sequences are located in the initial part of the gene (Figure 61).

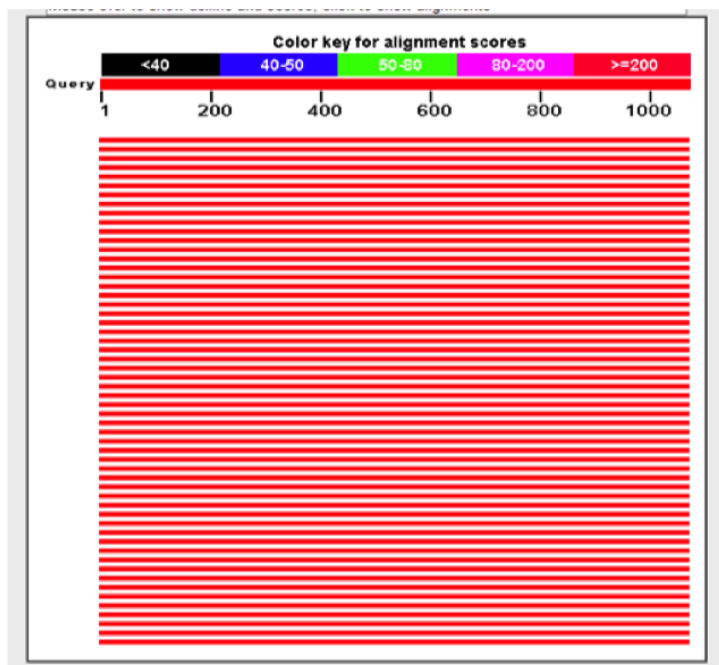


Figure 61 Nucleotide BLAST search of 18S obtained sequences. First hit is *Helianthus annuus*.

UBC 21 gene

For this gene only *L. vulgare* cDNA template provided significant results. Among the obtained sequences, “PT_79_1_T7.ab1” appears to be less *UBC* gene specific than “PT_79_4_T7.ab1” and “PT_79_18_T7.ab1”. The reason is that for “PT_79_1_T7.ab1” in position 1005 of original sequence there is a deletion of a single base, which gives a different open reading frame (ORF). PT_79_18 and PT_79_4 sequences are almost identical each other and could be used as reference for downstream experiments.

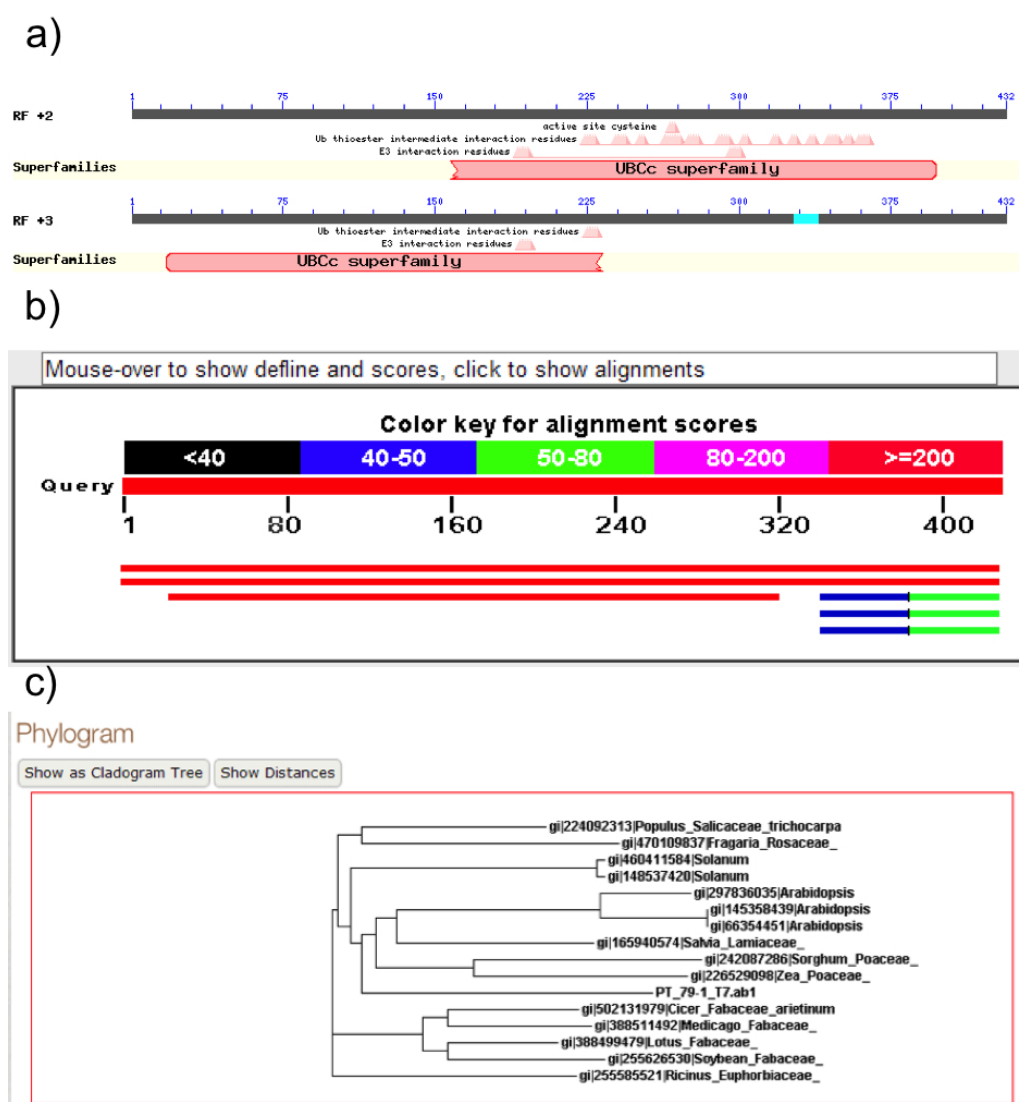


Figure 62 a) Protein domain search with BLASTx; b) Nucleotide BLASTn search; Phylogram of UBC gene family with the obtained sequences.

UBC 8-28 gene

Only *L. vulgare* cDNA template provided remarkable results. Both obtained sequences are specific to *UBC* domain, but a clear identification of the correspondence to *UBC_8* or *UBC_28* gene was not possible.

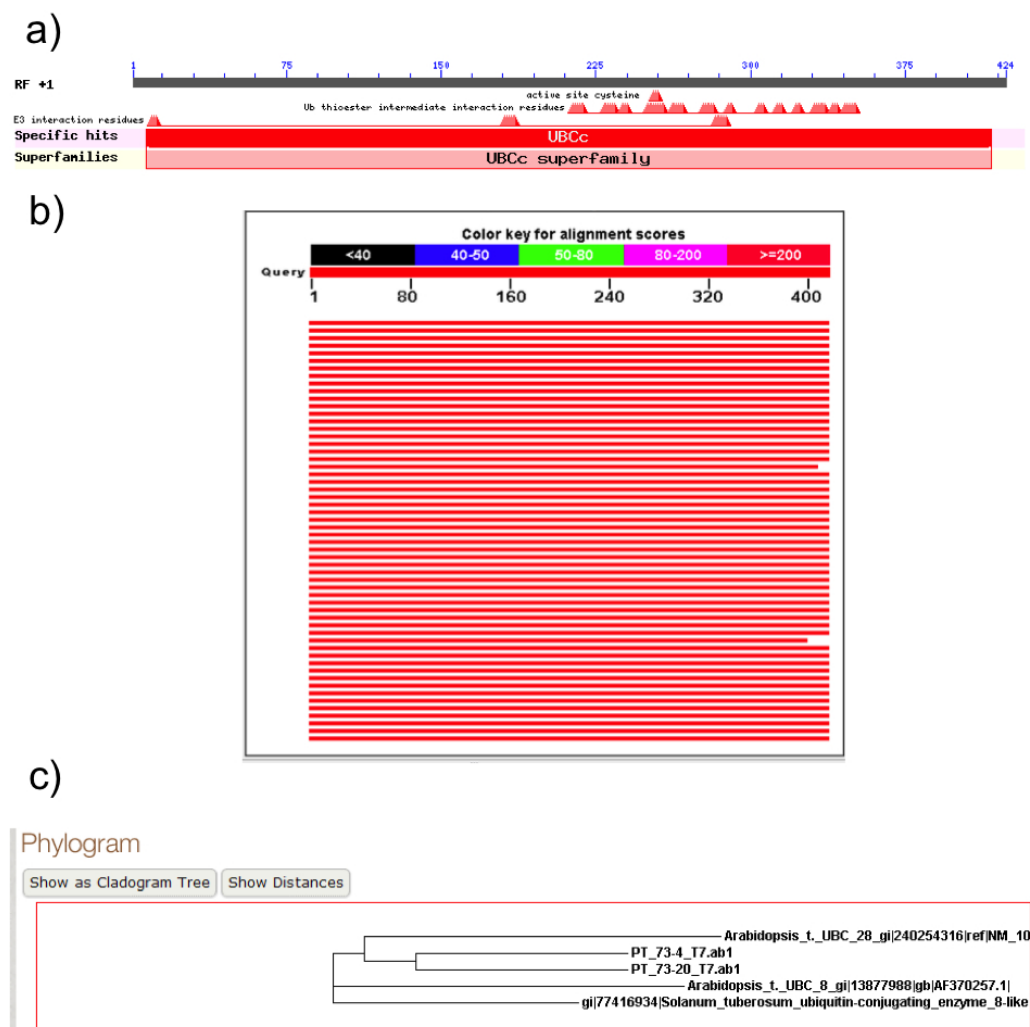


Figure 63 a) Protein domain search with BLASTx; b) Nucleotide BLASTn search; Phylogram of UBC gene family with the obtained sequences.

UBC 9-10 gene

For this gene all the obtained sequences were amplified from *L. vulgare* cDNA and they are very similar each other. In comparison with all the other *UBC* sequences, taken from NCBI database, they clustered together apart from other sequences.

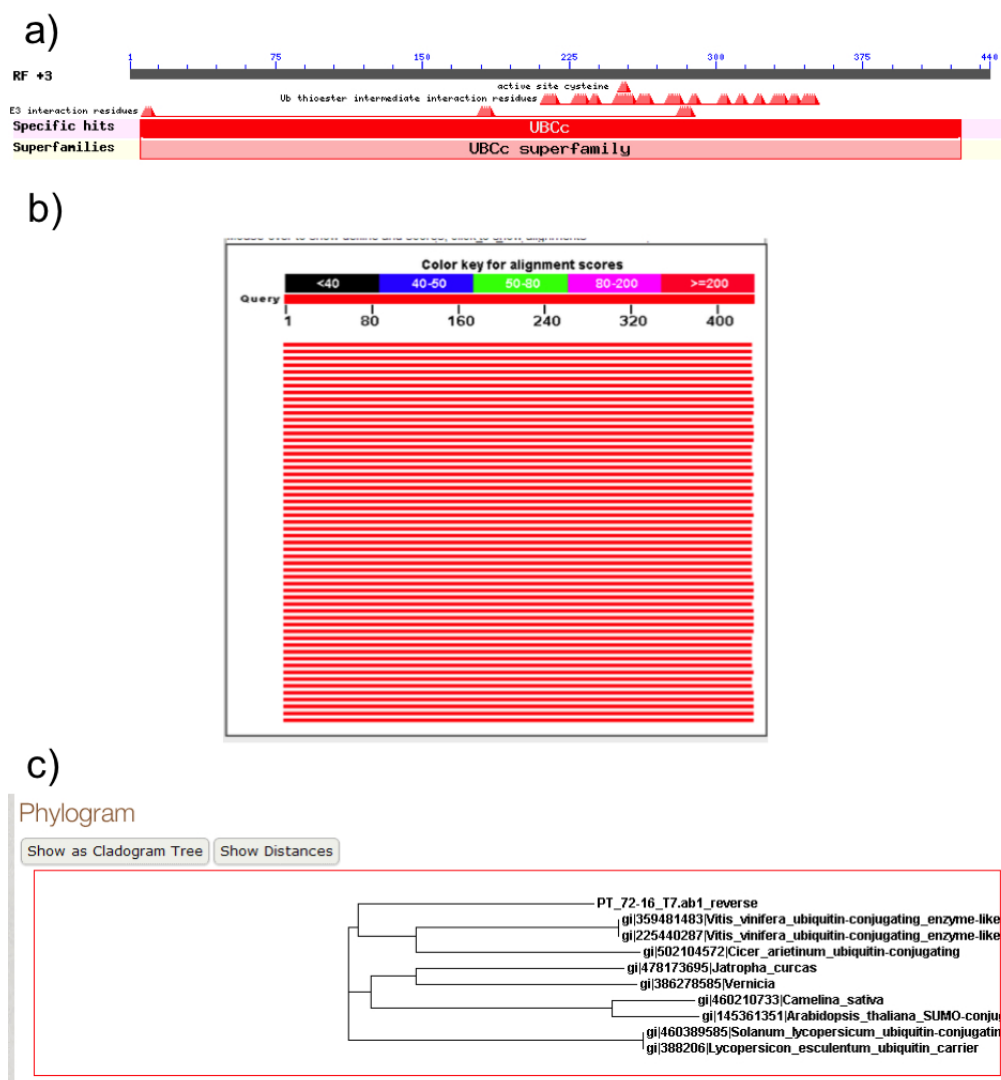


Figure 64 a) Protein domain search with BLASTx; b) Nucleotide BLASTn search; Phylogram of UBC gene family with the obtained sequences.

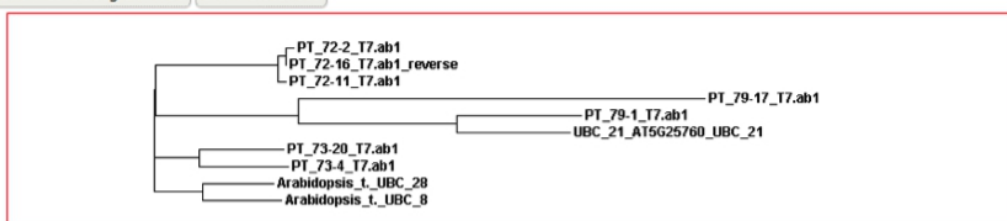
UBC sequences:

Only the amplicons obtained using *L. vulgare* cDNA as template gave significant results. In the following two phylograms (Figure 65) are reported which show that:

- PT_79 sequences are similar to *UBC_21* gene sequence as expected;
- PT_73 sequences are similar to *UBC_8* gene sequence and UBC 28 as expected;
- PT_72 sequences are similar to *UBC_9* and *UBC_10* gene sequences as expected.

a)

Phylogram



b)

Phylogram

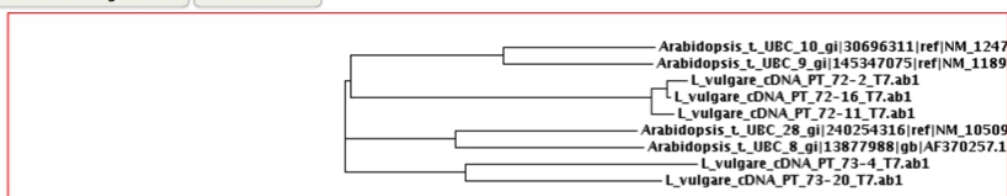


Figure 65 a) and b) phylograms of all the obtained UBC sequences compared with Arabidopsis sequences.

Expansin gene:

Both *C. coronarium* and *L. vulgare* sequences during BLASTx search matched *DPBB* domain, which is a conserved region that has the Double-Psi Beta-Barrel (DPBB) fold. Moreover another hit was the *PLN00050* domain, which is a provisional domain for *Expansin A*.

Sequences from both organism are similar each other. Alignment among *C. coronarium*, *L. vulgare* sequences and *Gerbera hybrid* and *Zinnia elegans* Expansin gene sequence showed that *C. coronarium* clustered apart from other sequences (Figure 66).

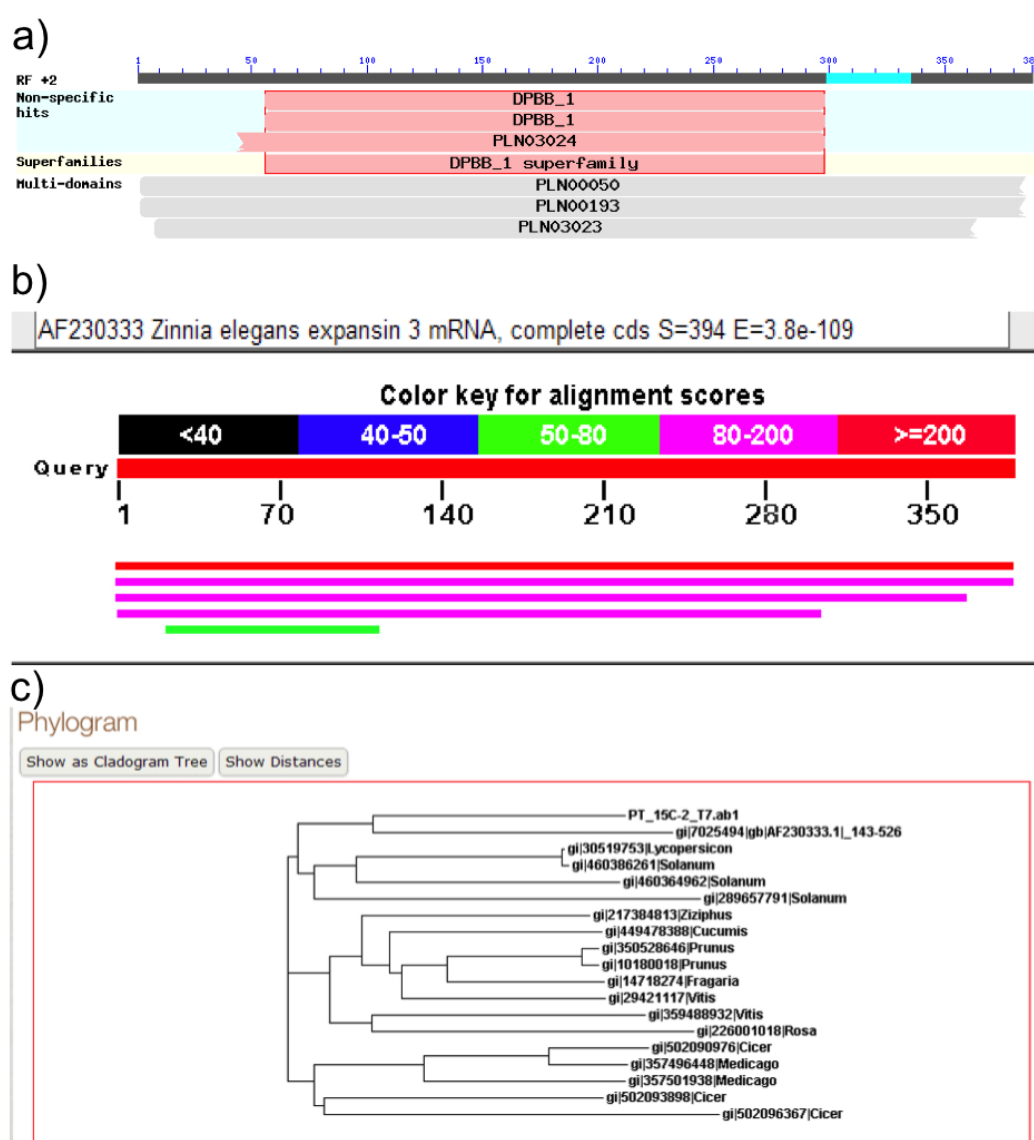


Figure 66 a)Protein domain search with BLASTx; b) Nucleotide BLASTn search; Phylogram of expansin gene family with the obtained sequences. GenBank: AF230333.1 is “*Zinnia elegans* expansin 3 mRNA, complete cds”.

Discussion

Chrysanthemum coronarium germination physiology

Investigation on germination behaviour of *Chrysanthemum coronarium* point out that this weed species is dormant as suggested earlier (F. M. Bastida 2004) (Banon 2009) (F. J. Bastida 2010). Although excised seeds from pericarp tissues fully germinated, anatomical observation of pericarp tissue showed the presence of brachysclereid cells with multiple pit canals by which water can flow during achene imbibition. In addition, imbibition curve of both morphotype showed clearly that covering seed structures does not prevent water uptake. So that, a physical dormancy, which, according to Baskin & Baskin seed dormancy classification (J. M. Baskin 2004), corresponds to the presence of specialized structures that avoid water entrance, appears to be unlikely. On the other hand, investigation on physiological dormancy release through several methodologies does not provided any incontrovertible evidence of a complete dormancy breaking. However, eight-weeks wet-warm stratified achenes at 35/20° C produced a significant increase of germinability (up to 55%) for both varieties in central and in peripheral achenes. So, the synergistic presence of water together with alternating high temperature could play a role in dormancy release in *Chrysanthemum coronarium*.

Furthermore, achenes tested with dry-storage conditions showed a dormancy release (up to 35% of germination).

Interestingly, in all the tested conditions fruit coat rupture always preceded germination this event occurred in a much more faster way in wet-warm stratified seeds then in other conditions. Also long term dry storage achenes resulted in a high percentage of fruit coat rupture. This could be explained with the action that seed coat may have in mechanical constraint to the radicle protrusion, due to the hard lignified pericarp. In particular, in peripheral seed the broader thickness of pericarp could increase their inability to promptly germinate when imbibed.

The type of dormancy operating in *Chrysanthemum coronarium* best fits the model of non-deep physiological dormancy. In seeds with this type of dormancy, fresh seeds fail to germinate unless they receive a dormancy-breaking treatment of some kind (e.g. cold or warm temperatures, chemicals, or light). Excised embryos (or seeds) grow into normal seedlings however.

Inhibition of germination mediated by seed surrounding structure is widely reported in literature often due to presence of inhibitors (K. V. Beneke 1992) (K. V. Beneke 1993), or thick pericarp (Tanowitz 1987), or blocking diffusion of gasses (McEvoy 1984).

Thus, observing the 40 and 45° C wet-warm treatment, the tentative conclusion that the wet-warm cue may have rapidly weakened the fruit coat is very interesting, and worthy of follow-up. The reason of this weakening may lie in a seed over-imbibition allowed by a stronger seed osmotic potential. Seed osmotic potential increase may originate from dormancy-release associated physiological mechanisms or just by a physical mechanism where, according to van't Hoff equation, an increase in temperature cause a slightly increase in ψ_{π} value.

In natural environment a probable mechanism through which dormancy is released could be through pericarp tissue weaken which is achieved after long time in soil (more prolonged for peripheral achenes than central ones) establishing, eventually, a robust seed bank in the soil.

***Chrysanthemum coronarium* varietal genotyping**

The two different approaches used revealed a very moderate level of genetic differentiation between the *C. coronarium* varieties. In particular, even though the DNA barcode methodology clearly indicated that varietal differentiation could not arose from inter species differentiation, the ISSR approach pointed out an unstructured diversity between *discolor* and *concolor*;

Moreover, germination behaviour, complying with genetic composition results, showed not significant differences within the tested varieties.

***Leucanthemum vulgare* germination physiology**

Leucanthemum vulgare is a non-dormant species and it is very fast in germination within a broad range of temperatures. Interestingly, the priming treatment causes a germination response inhibition at low temperature that appear to be a *dormancy-induction* process. After this dormancy induction treatment seed germination is inhibited at 10° C and the germination temperature range is moved towards higher temperatures.

Using longer priming periods the dormancy state starts to weaken. In particular, after the long priming treatment (nine-weeks priming) at 5° C dormancy seems to be completely released.

Incubation in darkness could have a role in dormancy induction because seed primed in light especially at 10° produces a final germination percentage higher than seeds primed in dark with the same conditions. Low water potential is also involved in the germination inhibition causing a decrease of 29% when compared with control at 10° C.

Furthermore, gibberellic acid added to 5 weeks primed seeds caused an increase of 20% in germination frequency.

On the other hand, fluridone addition during one or five-weeks (data not shown) priming treatment did not prevented the dormancy induction process. While when one-week primed seeds were imbibed with 50 µM of fluridone it produced a significant germination increase (about 40%) suggesting that, at this stage, ABA synthesis could have a role in dormancy maintenance.

This discrepancy in germinability in response to fluridone addition, could be explained supposing that the dormancy induction during priming is not an ABA mediated process, while, later, ABA mediates the maintenance of dormancy, as widely investigated in literature. Conversely, the dormancy induction process could be produced by a synergistic effect of low water osmotic potential and darkness.

Cloning of reference and dormancy associated genes:

Five probable housekeeping genes *18S* rRNA, *Phosphoglycerate kinase (PGK)* and *UBC_8*, *UBC_9*, *UBC_21* in *Leucanthemum vulgare* and in *Chrysanthemum coronarium* var. *concolor* were selected, amplified, cloned and identified suitable for normalizing real-time PCR data. This is the first study to identify the appropriate reference genes in these two weed species. Subsequent examination of expression level of these isolated genes could validate as reliable genes for reference in quantitative PCR studies.

With the same methodology *Expansin* gene was isolated in *Leucanthemum vulgare* and in *Chrysanthemum coronarium* species. *DOG1*, *FLC* and *HUB2* genes isolation is underway. The expression profile of these genes will be analysed during different developmental stage of seeds, natural dormancy, and induction of dormancy. This will unveil their relationship with the molecular regulation of dormancy.

Conclusions

In this work the germination physiology of *Chrysanthemum coronarium* var. *concolor* and *discolor* was investigated and the conditions for dormancy release were identified. This finding gives a substantial contribution to the knowledge of seed germination in *Chrysanthemum coronarium* for which relatively few studies are available. Further studies are required to assess the specific mechanism of dormancy release during warming treatment, how this is related to fruit coat weakening and if this would be associated to a change in seed osmotic potential.

Moreover, systems for dormancy induction and dormancy release were identified in a non-dormant species, *Leucanthemum vulgare*, which could be used as reference weed species in further comparative studies on dormancy regulation mechanisms.

In addition, isolation of reference genes to be used in quantitative PCR analysis together with the identification of dormancy-associated genes in these two species would be an essential tool for gene expression analysis of dormancy establishment and its maintenance.

Reference cited

- Amen, R.D. “A model of seed dormancy.” *Botanical Review* 34 (1968): 1-31.
- Amritphale, D., Dixit S., Singh B. “Effect of acetone on the induction and breakage of secondary dormancy in seeds of cucumber.” *Journal of Experimental Botany* 44 (1993): 1621-1626.
- Amritphale, D., Sreenivasulu Y., Singh B. “Changes in Membrane Fluidity and Protein Composition during Release of Cucumber Seeds from Dormancy by a Higher Temperature Shift.” *Annals of Botany* 85 (2000): 13-18.
- Andersson, L., Milberg, P. “Variation in seed dormancy among mother plants, populations and years of seed collection.” *Seed science research* 8 (1998): 29-38.
- Arif, M., Jan M. T., Marwat K. B., Khan M. A. “Seed priming improves emergence and yield of soybean.” *Pakistan Journal of Botany* 40, no. 3 (2008): 1169-1177.
- Ascher, P. “Self-incompatibility systems in floriculture crops.” *Acta Horticulturae* 63 (1976): 205-216.
- Ashraf, M., Foolad R.M. “Pre-sowing seed treatment—a shotgun approach to improve germination, plant growth and crop yield under saline and non-saline conditions.” *Advances in Agronomy* 88 (2005): 223-271.
- Augspurger, C.K. “Seed dispersal of the tropical tree *Platypodium elegans* and the escape of its seedlings from fungal pathogens.” *Journal of Ecology* 71 (1983): 759-771.
- Banon, S., Martinez-Sanchez, J.J., Vicente, M.J., Conesa, E., Franco, J.A., Fernandez, J.A. “Effect of applying commercial gibberellins on seed germination of *Chrysanthemum coronarium* L.” *Acta horticulturae* 813 (2009): 435-439.
- Barcaccia, G., F. Arzenton, T. F. Sharbel, S. Varotto, P. Parrini, and M. Lucchin. “Genetic diversity and reproductive biology in ecotypes of the facultative apomict *Hypericum perforatum* L.” *Heredity* 96 (2006): 322–334.
- Baskin, C. C. “Breaking physical dormancy in seeds – focussing on the lens.” *New Phytologist* 158, no. 2 (2003): 229-232.
- Baskin, C. C., J. M. Baskin. *Seeds: ecology biogeography and evolution of dormancy and germination*. San Diego: Academic Press, 1998.
- Baskin, C. C., J. M. Baskin, A. Yoshinaga, K. Thompson. “Germination of drupelets in multi-seeded drupes of the shrub *Leptecophylla tameiameia* (Ericaceae) from Hawaii: a case for deep physiological dormancy broken by high temperatures.” *Seed Science Research* 15, no. 4 (2005): 349-356.
- Baskin, J. M., C. C. Baskin. “A classification system for seed dormancy.” *Seed Science Research* 14, no. 1 (2004): 1-16.
- Baskin, J. M., C. C. Baskin. “Effect of chilling and giberellic acid on growth potential of excised embryos of *Ruellia humilis*.” *Planta* 100 (1971): 365-369.
- Baskin, J. M., C. C. Baskin. “Germination ecology of *Phacelia dubia* var. *dubia* in Tennessee glade.” *American Journal of Botany* 58 (1971): 98-104.
- Baskin, J. M., C. C. Baskin. “Role of temperature in the germination ecology of three summer annual weeds.” *Oecologia* 30 (1977): 377-382.
- Baskin, J. M., C. C. Baskin. “Temperature requirement for after-ripening in buried seeds of four summer annual weeds.” *Weed Research* 27 (1987): 385-389.
- Baskin, J. M., C. C. Baskin. “Temperature requirements for afterripening of seeds of a winter annual induced into secondary dormancy by low winter temperatures.” *Bulletin of the Torrey Botanical Club* 105 (1978): 104-107.

- Baskin, J. M., C. C. Baskin, L. Xiaojie. “Taxonomy, anatomy and evolution of physical dormancy in seeds.” *Plant Species Biology* 15, no. 2 (2000): 139-152.
- Baskin, J. M., X. Nan, C. C. Baskin. “A comparative study of seed dormancy and germination in an annual and a perennial species of *Senna* (Fabaceae).” *Seed Science Research* 8 (1998): 501–512.
- Baskin, J.M., Baskin, C.C. “Germination dimorphism in *Heterotheca subaxillaris* var. *subaxillaris*.” *Bulletin of the Torrey Botanical Club* 103 (1976): 201-206.
- Basra, S. M. A., Farooq M., Tabassum R. “Physiological and biochemical aspects of seed vigour enhancement treatments in fine rice (*Oryza sativa* L.).” *Seed Science Technology* 33 (2005): 623–628.
- Basra, S. M. A., Pannu I. A., Afzal I. “Evaluation of seedling vigor of hydro and matriprimed wheat (*Triticum aestivum* L.) seeds.” *International Journal of Agricultural Biology* 5 (2003): 121-123.
- Bassel, G. W., Lan, H., Glaab, E., Gibbs, D. J., Gerjets, T., Krasnogor, N., Bonner A. J., Holdsworth, M. J., Provart, N. J. “Genome-wide network model capturing seed germination reveals coordinated regulation of plant cellular phase transitions.” *Proceedings of Natural Academy of Sciences U.S.A.* 108, no. 23 (2011): 9709-9714.
- Bastida, F., J.L.G. Andujar, F. J. Monteagudo, J. Menendez. “Aerial seed bank dynamics and seedling emergence patterns in two annual Mediterranean Asteraceae.” *Journal of Vegetation Science* 21 (2010): 541-550.
- Bastida, F., Menendez J. “Germination requirements and dispersal timing in two heterocarpic weedy Asteraceae.” *Communications in Agricultural and Applied Biological Sciences* 69 (2004): 67-76.
- Beltran-Petia, E., A. Ortiz-López, E. Sanchez de Jiménez. “Synthesis of ribosomal proteins from stored mRNAs early in seed germination.” *Plant Molecular Biology* 28 (1995): 327-336.
- Beneke, K., Van Rooyen M. W., Theron G. K. “Fruit polymorphism in ephemeral species of Namaqualand. IV. Growth analysis of plants cultivated from the dimorphic diaspores.” *Journal of Arid Environments* 24 (1993): 345–360.
- Beneke, K., Von Teichman I., Van Rooyen M. W., Theron G.K. “Fruit polymorphism in ephemeral species of Namaqualand. I. Anatomic differences between polymorphic diaspores of two *Dimorphotheca* species.” *South African Journal of Botany* 58 (1992): 448–455.
- Bennett, M. A., Waters L. “Seed hydration treatments for improved sweet maize germination and stand establishment.” *Journal American Society Horticultural Science* 112 (1987): 45–49.
- Bentsink, L., Hanson J., Hanhart C. J., Vries H. B., C. Coltrane, P. Keizer, M. El-Lithy, C. Alonso-Blanco, M. T. de Andrése, M. Reymond, F. vann Eeuwijk, S. Smekens, and M. Koornneef. “Natural variation for seed dormancy in *Arabidopsis* is regulated by additive genetic and molecular pathways.” *Proceedings of the National Academy of Sciences* 107, no. 9 (2010): 4264–4269.
- Bentsink, L., Jowett J., Hanhart J. C., Koornneef M. “Cloning of DOG1, a quantitative trait locus controlling seed dormancy in *Arabidopsis*.” *PNAS* 103, no. 45 (2006): 17042–17047.
- Bewley, J. D. “Seed Germination and Dormancy.” *The Plant Cell* 9 (1997): 1055-1066.
- Bewley, J. D., A. Marcus. “Gene expression in seed development and germination.” *Progress in Nucleic Acid Research and Molecular Biology* 38 (1990): 165-193.
- Bewley, J. D., M. Black. *Seeds: Physiology of Development and Germination*. New York: Plenum Press, 1994.

- Bhattacharyya, D. “Karyomorphological studies in some species of the genus *Chrysanthemum*.” *Science and Culture* 43 (1977): 431-432.
- Bhowmik, P. C. “Weed biology: importance to weed management.” *Weed science* 45 (1997): 349-356.
- Blaxter, M. L. “The promise of a DNA taxonomy.” *Philos Trans R Soc Lond B Biol Sci* 359, no. 1444 (2004): 669-679.
- Blumenthal, A., Lerner, H.R., Werker, E., Poljakoff-Mayber, A. “Germination preventing mechanisms in *Iris* seeds.” *Annals of Botany* 58 (1986): 551-561.
- Borner, B., Branchard M. “Non anchored inter simple sequence repeat (ISSR) markers: reproducible and specific tools for genome fingerprinting.” *Plant Molecular Biology RepORT* 19 (2001): 209-215.
- Botha, F.C., Potgieter, G.P., Botha, A.M. “Respiratory metabolism and gene expression during seed germination.” *Journal of Plant Growth Regulation* 11 (1992): 211-224.
- Brändel, M. “Dormancy and germination of heteromorphic achenes of *Bidens frondosa*.” *Flora* 199 (2004): 228–233.
- Brändel, M. “Ecology of achene dimorphism in *Leontodon saxatilis*.” *Annals of Botany* 100 (2007): 1189–1197.
- Bradford, K. J. “Manipulation of seed water relations via osmotic priming to improve germination under stress conditions.” *Horticultural Science* 21 (1986): 1105-1112.
- Bray, C. M., Davison P. A., Ashraf M., Taylor M. R. “Biochemical events during osmopriming of leek seed.” *Annals of Applied Biology* 102 (1989): 185-193.
- Bremer, K., Humphries, C. J. *The generic monograph of Asteraceae-Anthemideae*. . Vol. 23. London: Bull. Nat. History Mus., 1993.
- Bruggink, G. T., Ooms J.J.J., Vander Toorn P. “Induction of longevity in primed seeds.” *Seed Science Research* 9 (1999): 49-53.
- Brullo, S., Costanzo E., Tomaselli V. “Phytosociological study on the *Laurus* communities in the Hyblaean Montains (SE Sicily).” *Phytocoenologia* 31, no. 2 (2001): 249-170.
- Brullo, S., Marcenò C. “Contributo alla conoscenza della vegetazione nitrofila della Sicilia.” *Coll. Phytos.* 12 (1985): 23-148.
- Cadman, C. S. C., Toorop, P. E., Hilhorst, H. W. M., Finch-Savage, W. E. “Gene expression profiles of *Arabidopsis* Cvi seeds during dormancy cycling indicate a common underlying dormancy control mechanism.” *Plant Journal* 46 (2006): 805-822.
- Cano, E., C. M. Musarella, A. Cano-Ortiz, C. J. Pinto Gomes, G. Spampinato, G. Ferro, J. A. Garrido, J. Mota. “Analysis of the *Chrysanthemum coronarium* communities in the Mediterranean.” *Gestao e Conservacao da Biodiversidade*. Tortosendo, 2012. 56-57.
- Cano, E., C.M. Musarella, G. Spampinato, J. C. Piñar Fuentes, C. J. Pinto Gomes. “Taxonomic analysis of *Glebionis coronaria* (L.) Cass. ex Spach in the Mediterranean area.” *VII International Seminar Management and Biodiversity Conservation “Planning and management of agricultural and forestry resources”*. Gambarie d’Aspromonte – RC (Italy),, 2013. 56.
- Cano-Ortiz, A., Cano, E., González, A. Cano-Ortiz, A. *Flora medicinal y aromática*. Malaga: Servicio de Publicaciones -Universidad de Jaén, 2007.
- Carpita, N. C., A. Sharia, J. P. Barnett, J. R. Dunlap. “Cold stratification and growth of radicles of loblolly pine (*Pinus taeda*) embryos.” *Physiologia Plantarum* 59 (1983): 601-606.

- Carr, G. D., R. M. King, A. M. Powell, H. Robinson. “Chromosome numbers in Compositae.” *American Journal of Botany* 86 (1999): 1003-1013.
- Carrera, E., Holman, T., Medhurst, A., Peer, W., Schmuths, H., Footitt, S., Theodoulou, F. L., Holdsworth, M. J. “Gene expression profiling reveals defined functions of the ATP-binding cassette transporter COMATOSE late in phase II of germination.” *Plant Physiology* 143, no. 4 (2007): 1669-1679.
- Casal, J. J., Sanchez, R. A. “Phytochromes and seed germination.” *Seed Science Research* 8 (1998): 317-329.
- Caseiro, R., Bennett M. A., Marcos-Filho J. “Comparison of three priming techniques for onion seed lots differing in initial seed quality.” *Seed Science Technology* 32 (2004): 365–375.
- Chen, F., Dahal P., Bradford K. J. “Two Tomato Expansin Genes Show Divergent Expression and Localization in Embryos during Seed Development and Germination.” *Plant Physiology* 127, no. 3 (2001): 928–936.
- Chen, K., Arora R. “Dynamics of the antioxidant system during seed osmopriming, post-priming germination, and seedling establishment in spinach (*Spinacia oleracea*).” *Plant Science* 180 (2011): 212–220.
- Chen, S.S.C. “Germination of light inhibited seed of *Nemophila insignis*.” *American Journal of Botany* 55 (1968): 1177-1183.
- Chiang G. C., Barua, D., Dittmar, E., Kramer, E. M., de Casas, R. R., Donohue, K. “Pleiotropy in the wild: the dormancy gene *DOG1* exerts cascading control on life cycles.” *Evolution* 67 (2013): 883-893.
- Chiang, G. C. K., Barua D., Kramera E. M., R. M. Amasinob, and K. Donohue. “Major flowering time gene, *FLOWERING LOCUS C*, regulates seed germination in *Arabidopsis thaliana*.” *Proceedings of the National Academy of Sciences* 106, no. 28 (2009): 11661-11666.
- Chiang, G. C., Bartsch, M., Barua, D., Nakabayashi, K., Debieu, M., Kronholm, I., Koornneef, M., Soppe, W. J., Donohue, K, De Meaux, J. “*DOG1* expression is predicted by the seed-maturation environment and contributes to geographical variation in germination in *Arabidopsis thaliana*.” *Molecular Ecology* 16 (2011): 3336-3349.
- Chiang, M.H., Park, K.W. “Effects of temperature, light and mechanical treatment on seed germination of *Chrysanthemum coronarium* L.” *Journal of Korean Society Horticultural Sciences* 35, no. 6 (1994): 534-539.
- Clark, W. S., Beattie, D. J., Arteca, R. N. “A Growth Inhibitor in *Clematis viticella* L. seeds.” *Journal of Plant Physiology* 134, no. 4 (1989): 492–495.
- Clavijo, E. Ruiz De. “The role of dimorphic achenes in the biology of the annual weed *Leontodon longirostris*.” *Weed research* 41 (2001): 275-286.
- Cockshull, K.E. “The photoperiodic induction of flowering in short-day plants.” In *Light and the flowering process*, by Thomas B., Cockshull K.E. Prue V., 33-50. London: Academic Press , 1984.
- Comai, L., Harada, J.J. “Transcriptional activities in dry seed nuclei indicate the timing of the transition from embryogeny to germination.” *Proceedings Natural Academy Science* 87 (1990): 2671-2674.
- Cook, R., Talley, T.S., Dayton, P., Wang, M., Nguyen, K. “The effect of invasive crown daisy (*Chrysanthemum coronarium*) on a coastal sage scrub arthropod community in Southern California.” *California Invasive Plant Council Symposium*. 2011. 63-65.

- Corbineau, F., Bagniol, S., Côme, D. “Sunflower (*Helianthus annuus* L.) seed dormancy and its regulation by ethylene.” *Israel Journal of Botany* 39, no. 4-6 (1990): 313-325.
- Corkid, L., Rincon, E., Vazquez-Yanes, C. “Effect of light and temperature on germination of heteromorphic achenes of *Bidens odorata* (Asteraceae).” *Canadian Journal of Botany* 69 (1991): 574–579.
- Cosgrove, D.J. “Relaxation in a high-stress environment: The molecular bases of extensible cell walls and cell enlargement.” *Plant Cell* 9 (1997): 1031-1041.
- Crowe, J.H., L.M. Crowe. “1992). Membrane integrity in anhydrobiotic organisms: Toward a mechanism for stabilizing dry seeds.” In *Water and Life*, by C.B. Osmond, and C.L. Bolis G.N. Somero, 87-103. Berlin: Springer-Verla, 1992.
- Cullings, K. W. “Design and testing of a plant-specific PCR primer for ecological and evolutionary studies.” *Molecular Ecology* 1 (1992): 233-240.
- Czechowski, T., Stitt M., Altmann T., Udvardi M.K., Scheible W.R. “Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*.” *Plant Physiology* 139, no. 1 (2005): 5-17.
- Dekkers, S.J.W., Willems L., Bassel G.W., van Bolderen-Veldkamp R.P., Ligterink W., Hilhorst H.W.M., and Bentsink L. “Identification of reference genes for RT-qPCR expression analysis in *Arabidopsis* and tomato seeds.” *Plant Cell Physiology* 53 (2011): 28-37.
- d’Urville, Dumont. *Mémoire Société Linnéenne* 1 (1822): 368.
- Dogan, B., A. Duran, E. E. Hakki. “Phylogenetic analysis of *Jurinea* (Asteraceae) species from Turkey based on ISSR amplification.” *Ann. Bot. Fennici* 44 (2007): 353-358.
- Dommès, J., C. Van de Walle. “Polysome formation and incorporation of new ribosomes into polysomes during germination of the embryonic axis of maize.” *Physiologia Plantarum* 79 (1990): 289-296.
- Donohue, K., de Casas, R. R., Burghardt L., Kovach K., Willis C. G. “Germination, Postgermination Adaptation, and Species Ecological Ranges.” *Annual Review of Ecology, Evolution, and Systematics* 41 (2010): 293–319.
- Douglas, G. W., G. B. Straley, D. V. Meidinger, J. Pojar. *Illustrated Flora of British Columbia*. British Columbia, 1998.
- Dowrick, G.J. “The chromosomes of *Chrysanthemum*, I: The species.” *Heredity* 6 (1952): 365-367.
- Doyle, J. J., and E. E. Dickson. “Preservation of plant samples for DNA restriction endonuclease analysis.” *Taxon* 36 (1987): 715-722.
- Doyle, J. J., and J. L. Doyle. “A rapid DNA isolation procedure for small quantities of fresh leaf tissue.” *Phytochemistry Bulletin* 19 (1987): 11-15.
- Durán, J. M., Retamal, N. “Coat Structure and Regulation of Dormancy in *Sinapis arvensis* L. seeds.” *Journal of Plant Physiology* 135, no. 2 (1989): 218–222.
- Ebach, M.C., Holdrege C. “DNA Barcoding is no substitute for taxonomy.” *Nature* 434 (2005): 697.
- El-Twab A. M. H., Zahran F. A. “RAPD, ISSR and RFLP Analysis of Phylogenetic Relationships Among Congeneric Species (Anthemideae, Asteraceae) in Egypt.” *International Journal of Botany* 6, no. 1 (2010): 1-10.
- El-Twab, M.H.A., Mekawy, A.M., El-Katatny, M.S. “Karyomorphological studies of some species of *Chrysanthemum sensu lato* in Egypt.” *Chromosome Botany* 3 (2008): 41-47.

- Farooq, M., Basra S. M. A., Wahid A. “Priming of field sown rice seed enhances germination, seedling establishment, allometry and yield.” *Plant Growth Regulation* 44 (2006): 285-294.
- Fenner, M., Thompson, K. *The Ecology of Seeds*. Cambridge: Cambridge University Press, 2005.
- Ferro, G. “La vegetazione di Butera (Sicilia Meridionale).” *Atti Ist. bot. Univ. Lab. critt. Pavia* 13 (1980): 51-118.
- Ferro, G. “Nuovi dati sulla flora e sulla vegetazione dei coltivi e degli incolti di Lipari (Isole Eolie).” *Quad. Bot. Amb. Appl.* 15 (2004): 21-39.
- Finch-Savage, W. E., C.S.C. Cadman, P. E. Toorop, J. R. Lynn, H. W. M. Hilhorst. “Seed dormancy release in *Arabidopsis* Cvi by dry after-ripening, low temperature, nitrate and light shows common quantitative patterns of gene expression directed by environmentally specific sensing.” *Plant Journal* 51 (2007): 60-78.
- Finch-Savage, W. E., Dent K. C., Clark L.J. “Soak conditions and temperature following sowing influence the response of maize (*Zea mays* L.) seeds to on-farm priming (pre-sowing seed soak).” *Field Crops Research* 90 (2004): 361-374.
- Finch-Savage, W. E., Footitt, S. “To germinate or not to germinate: a question of dormancy relief not germination stimulation.” *Seed Science Research* 22, no. 4 (2012): 243-248.
- Finch-Savage, W. E., J. H. W. Bergervoet, R. J. Bino, H. A. Clay, S. P. C. Groot. “Nuclear Replication Activity During Seed Development, Dormancy Breakage and Germination in Three Tree Species: Norway Maple (*Acer platanoides* L.), Sycamore (*Acer pseudoplatanus* L.) and Cherry (*Prunus avium* L.).” *Annals of Botany* 81, no. 4 (1998): 519-526.
- Finch-Savage, W. E., Leubner-Metzger G. “Seed dormancy and the control of germination.” *New Phytologist* 171 (2006): 501-523.
- Finkelstein, R., Reeves, W., Ariizumi, T., Steber, C. “Molecular aspects of seed dormancy.” *Annual Review Plant Biology* 59 (2008): 387-415.
- Footitt, S., Clay, H. A., Dent, K., Finch-Savage, W. E. “Environment sensing in spring-dispersed seeds of a winter annual *Arabidopsis* influences the regulation of dormancy to align germination potential with seasonal changes.” *New Phytologist*, 2014.
- Footitt, S., Douterelo-Soler, I., Clay, H., Finch-Savage, W. E. “Dormancy cycling in *Arabidopsis* seeds is controlled by seasonally distinct hormone-signaling pathways.” *Proceedings of the National Academy of Sciences* 108, no. 50 (2011): 20236–20241.
- Footitt, S., Huang, Z., Clay, H. A., Mead, A., Finch-Savage, W. E. “Temperature, light and nitrate sensing coordinate *Arabidopsis* seed dormancy cycling, resulting in winter and summer annual phenotypes.” *The Plant Journal* 74 (2013): 1003–1015.
- Forsyth C., Brown N.A.C.,. “Germination of the dimorphic fruits of *Bidens pilosa* .” *New Phytologist* 90 (1982): 151–164.
- Gallardo, K., C. Job, S. P.C. Groot, M. Puype, H. Demol, J. Vandekerckhove, and D. Job. “Proteomics of *Arabidopsis* seed germination: A comparative study of wild-type and gibberellin-deficient seeds.” *Plant Physiology* 129 (2002): 823-837.
- Graeber, K., Voegelé, A., Büttner-Mainik, A., Sperber, K., Mummenhoff, K., Leubner-Metzger, G. “Spatiotemporal Seed Development Analysis Provides Insight into Primary Dormancy Induction and Evolution of the *Lepidium* DELAY OF GERMINATION 1 Genes.” *Plant Physiology* 161 (2013): 1903–1917.

- Guan, Y., Hu, J., Wang, X., Shao, C. “Seed priming with chitosan improves maize germination and seedling growth in relation to physiological changes under low temperature stress.” *Journal of Zhejiang Univ Sci B* 10, no. 6 (2009): 427–433.
- Gutterman, Y. “Do germination mechanisms differ in plants originating in deserts receiving winter or summer rain?” *Israel Journal of Botany* 39 (1990): 355-372.
- —. *Seed Germination in Desert Plants*. Berlin, 1993.
- Hamley, D. H. “Softening of the seeds of *Melilotus alba*.” *Botanical gazette* 93 (1932): 345-375.
- Han, B., D. W. Hughes, G. A. Galau, J. D. Bewley, A. R. Kermode. “Changes in late embryogenesis abundant (Lea) messenger RNAs and dehydrins during maturation and premature drying of *Ricinus communis* L. seeds.” *Planta* 201 (1996): 27-35.
- Harms, K. E., S. J. Wright, O. Calderon, A. Hernandez, E. A. Herre. “Pervasive density-dependent recruitment enhances seedling diversity in a tropical forest.” *Nature* 404 (2000): 493-494.
- Hilhorst, H.W.M., Finch-Savage, W.E., Buitink, J., Bolingue, W., Leubner-Metzger, G. *Dormancy in plant seeds*. Vol. 21, in *Dormancy and resistance in harsh environments*, by Lubzens E, 43-67. Topics in Current Genetics, 2010.
- Holdsworth, M. J., L. Bentsink, W. J. J. Soppe. “Molecular networks regulating Arabidopsis seed maturation, after-ripening, dormancy and germination.” *New Phytologist* 179 (2008): 33-54.
- Hollingsworth, P. M, Forrest L. L., Spouge J. L., Mehrdad Hajibabaei, Sujeevan Ratnasingham, Michelle van der Bank, Mark W. Chase, Robyn S. Cowan, David L. Erickson, Aron J. Fazekas, Sean W. Graham, Karen E. James, Ki-Joong Kim, W. John Kress, Harald Schneider, Jonathan van AlphenStahl, Spencer C.H. Barrett, Cassio van den Berg, Diego Bogarin, Kevin S. Burgess, Kenneth M. Cameron, Mark Carine, Juliana Chacón, Alexandra Clark, James J. Clarkson, Ferozah Conrad, Dion S. Devey, Caroline S. Ford, Terry A.J. Hedderson, Michelle L. Hollingsworth, Brian C. Husband, Laura J. Kelly, Prasad R. Kesanakurti, Jung Sung Kim, Young-Dong Kim, Renaud Lahaye, Hae-Lim Lee, David G. Long, Santiago Madriñán, Olivier Maurin, Isabelle Meusnier, Steven G. Newmaster, Chong-Wook Park, Diana M. Percy, Gitte Petersen, James E. Richardson, Gerardo A. Salazar, Vincent Savolainen, Ole Seberg, Michael J. Wilkinson, Dong-Keun Yi, and Damon P. Little. “A DNA barcode for land plants.” *Proceedings of the National Academy of Sciences* 106, no. 31 (2009): 12794-12797.
- Huangfu, C. h., X. l. Song, S. Qiang. “ISSR variation within and among wild Brassica juncea populations: implication for herbicide resistance evolution.” *Genet Resour Crop Evol* 56 (2009): 913-924.
- Hwang, H.J. “Improvement of In Vitro Seed Germination in Chicory (*Cichorium intybus* L.) and Garland Chrysanthemum (*Chrysanthemum coronarium* L.).” *Journal of Bio-Environment Control*, 2008: 297-305.
- Imbert, E. “Ecological consequences and ontogeny of seed heteromorphism. Perspectives in Plant Ecology.” *Perspectives in Plant Ecology, Evolution and Systematics* 5 (2002): 13-36.
- Imbert, E., Escarré J., Lepart J. “Differentiation among populations for life history, morphology, head traits and achene morph productions in the heterocarpic species *Crepis sancta* (Asteraceae).” *International Journal of Plant Sciences* 160 (1999): 543-552.

- Iqbal, M., Ashraf M. “Changes in growth, photosynthetic capacity and ionic relations in spring wheat (*Triticum aestivum* L.) due to presowing seed treatment with polyamines.” *Plant Growth Regulation* 46 (2005): 19–30.
- Jain, A., Bhatia S., Banga S. S., Prakash S., Lakshmikumaran M. “Potential use of random amplified polymorphic DNA (RAPD) technique to study the genetic diversity in Indian mustard (*Brassica juncea*) and its relationship to heterosis.” *Theoretical Applied Genetics* 88 (1994): 116–122.
- Janzen, D. H. “Seed predation by animals.” *Annual review of Ecology and Systematics* 2 (1971): 465-492.
- Janzen, D. H., Hajibabaei M., Burns J. M., Hallwachs W., Remigio, E. and Hebert, P. D. N. “Wedding biodiversity inventory of a large and complex Lepidoptera fauna with DNA barcoding.” *Philosophical Transactions of the Royal Society of London* 360 (2005): 1835-1845.
- Jauzein, P. *Flore des Champs Cultivés*. 2011.
- Jett, L. W., Welbaum G. E., Morse R. D. “Effect of matric and osmotic priming treatments on broccoli seed germination.” *Journal of American Society Horticultural Science* 121 (1996): 423-429.
- Jiang, L., A. R. Kermode. “Role of desiccation in the termination of expression of genes for storage proteins.” *Seed Science Research* 4 (1994): 149-173.
- Jisha, K. C., K. Vijayakumari, J. T. Puthur. “Seed priming for abiotic stress tolerance: an overview.” *Acta Physiologia Plantarum* 35 (2013): 1381–1396.
- Juntilla, O. “The mechanism of low temperature dormancy in mature seeds of *Syringa* species.” *Physiologia Plantarum* 29 (1973): 256-263.
- Kaul M.L., Kaur J. “Checkered prophase I in crown daisy.” *Cytologia* 60 (1995): 195-203.
- Kaur, S., Gupta A. K., Kaur N. “Effect of osmo- and hydropriming of chickpea on seedling growth and carbohydrate metabolism under water deficit stress.” *Plant Growth Regulation* 37 (2002): 17-22.
- Kaur, S., Gupta A. K., Kaur N. “Seed priming increases crop yield possibly by modulating enzymes of sucrose metabolism in chickpea.” *Journal of Agronomical Crop Science* 19 (2005): 81-87.
- Kaya, M. D., Okcu G., Atak M., Cikili Y., Kolsarici O. “Seed treatments to overcome salt and drought stress during germination in sunflower (*Helianthus annuus* L.)” *European Journal of Agronomy* 24 (2006): 291-295.
- Keller, M., Kollmann J. “Germination under controlled and natural conditions.” *Ecosystems and Environment* 72, no. 1 (1999): 87-99.
- Kelly, K. M., Van Staden, J., Bell, W. E. “Seed coat structure and dormancy.” *Plant Growth Regulation* 11 (1992): 201-209.
- Kendall, S. L., Hellwege, A., Marriot, P., Whalley, C., Graham, I. A., Penfield, S. “Induction of dormancy in *Arabidopsis* summer annuals requires parallel regulation of DOG1 and hormone metabolism by low temperature and CBF transcription factors.” *The Plant Cell* () 23, no. 7 (2011): 2568-2680.
- Khan, A. A., C. Samimy. “Hormones in relation to primary and secondary seed dormancy.” In *The physiology and biochemistry of seed development*, by Khan A.A., 203-241. Amsterdam/New York/Oxford: Elsevier, 1982.
- Khuroo, A.A., Malik, A.H., Reshi, Z.A., Dar, G.H. “From ornamental to detrimental: plant invasion of *Leucanthemum vulgare* Lam. (Ox-eye Daisy) in Kashmir valley, India.” *Current Science* 98, no. 5 (2010): 601-602.
- Kilian, J., D. Whitehead, J. Horak, D. Wanke, S. Weinl, O. Batistic, C. D'Angelo, E. Bornberg-Bauer, J. Kudla, K. Harter. “The AtGenExpress global stress expression

- data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses.” *Plant Journal* 50 (2007): 347-363.
- Kocik, A. 1996.
 - Koonjul, P. K., W. F. Brandt, J. M. Farrant, and G. G. Lindsey. “Inclusion of polyvinylpyrrolidone in the polymerase chain reaction reverses inhibitory effects of polyphenolic contamination of RNA.” *Nucleic Acids Research* 27, no. 3 (1999): 915-916.
 - Koornneef, M., Bentsink, L., Hilhorst, H. “Seed dormancy and germination.” *Current Opinion in Plant Biology* 5, no. 1 (2002): 33-36.
 - Kucera, B., Cohn, M.A., Leubner-Metzger, G. “Plant hormone interactions during seed dormancy release and germination.” *Seed Science Research* 15, no. 4 (2005): 281-307.
 - Kumar, K., M. Muthamilarasan, M. Prasad. “Reference genes for quantitative Real-time PCR analysis in the model plant foxtail millet (*Setaria italica* L.) subjected to abiotic stress conditions.” *Plant Cell Tissue and Organ Culture*, 2010.
 - Lahaye, R., Van der Bank M., Bogarin D., Warner J., Pupulin F., Gigot G., Maurin O., Duthoit S., Barraclough T. G., Savolainen V. “DNA barcoding the floras of biodiversity hotspots.” *Proceedings of the National Academy of Sciences* 105, no. 8 (2008): 2923–2928.
 - Lalonde, L., J. D. Bewley. “Desiccation during germination and its effects on the messenger RNA population.” *Experimental Botany* 37 (1986): 754-764.
 - Lane, B.G. “Cellular desiccation and hydration: Developmentally regulated proteins, and the maturation and germination of seed embryos.” *FASEB Journal* 5 (1991): 2893-2901.
 - Larkin, M. A., Blackshields G., Brown N.P., Chenna R., McGettigan P.A., McWilliam H., Valentin F., Wallace I.M., Wilm A., Lopez R., Thompson J.D., Gibson T.J. and Higgins D.G. “ClustalW and ClustalX version 2.” *Bioinformatics* 23, no. 21 (2007): 2947-2948.
 - Lauber, K., G. Wagner. *Flora Helvetica*. Berna, 2001.
 - Le Page-Degivry, M. T., Barthe, P., Garelo, G. “Involvement of Endogenous Abscissic Acid in Onset and Release of *Helianthus annuus* Embryo Dormancy.” *Plant Physiology* 92 (1990): 1164-1168.
 - Lee, C. R., Mitchell-Odds T. “Quantifying effects of environmental and geographical factors on patterns of genetic differentiation.” *Molecular Ecology* 20, no. 22 (2011): 4631-4642.
 - Lem, N.W., J.P. Williams,. “Desaturation of fatty acids associated with monogalactosyl diacylglycerol: The effect of SAN 6706 and SAN 9785.” *Plant Physiology* 68 (1981): 944-949.
 - Leubner-Metzger, G. “ β -1,3-Glucanase gene expression in low-hydrated seeds as a mechanism for dormancy release during tobacco after-ripening .” *The Plant Journal* 41 (2005): 133-145.
 - Leubner-Metzger, G. “Brassinosteroids and gibberellins promote tobacco seed germination by distinct pathways.” *Planta* 213 (2001): 758–763.
 - Leubner-Metzger, G. “Functions and regulation of β -1,3-glucanase during seed germination, dormancy release and after-ripening.” *Seed Science Research* 13 (2003): 17-34.
 - Leubner-Metzger, G., Frundt, C., Vogeli-Lange, R., Meins, F. “Class I [β]-1,3-Glucanases in the Endosperm of Tobacco during Germination.” *Plant Physiology* 109 (1995): 751-759.

- Leubner-Metzger, G., Meins, F. Jr. “Antisense-transformation reveals novel roles for class I β -1,3-glucanase in tobacco seed after-ripening and photodormancy.” *Journal of Experimental Botany* 52 (2001): 1753–1759.
- Levin S., D. Cohen, A. Hastings. “Dispersal strategy in patchy environments.” *Theoretical and Population Biology* 26 (1984): 165–191.
- Lin, J. M., Sung, J. M. “Pre-sowing treatments for improving emergence of bitter melon seedlings under optimal and sub-optimal temperatures.” *Seed Science and Technology* 29 (2001): 39–50.
- Linkies, A., Graeber, K., Knight, C., Leubner-Metzger, G. “The evolution of seeds.” *New Phytologist* 186, no. 4 (2010): 817–831.
- Linkies, A., Müller, K., Morris, K., Turečková, V., Wenk, M., Cadman, C.S.C., Corbineau, F., Strnad, M., Lynn, J.R., Finch-Savage, W.E., Leubner-Metzger, G. “Ethylene interacts with abscisic acid to regulate endosperm rupture during germination: A comparative approach using *Lepidium sativum* and *Arabidopsis thaliana*.” *The Plant Cell* 21 (2009): 3803–3822.
- Liu, Y., Koornneef, M., Soppe, W.J. “The absence of histone H2B monoubiquitination in the *Arabidopsis* hub1 (rdo4) mutant reveals a role for chromatin remodeling in seed dormancy.” *The Plant Cell* 19 (2007): 433–444.
- Magneschi, L., Kudahettigea, R.L., Alpi, A., Perata, P. “Expansin gene expression and anoxic coleoptile elongation in rice cultivars.” *Journal of Plant Physiology* 166 (2009): 1576–1580.
- Manz, B., K. Müller, B. Kucera, F. Volke, G. Leubner-Metzger. “Water Uptake and Distribution in Germinating Tobacco Seeds Investigated in Vivo by Nuclear Magnetic Resonance Imaging.” *Plant Physiology* 138 (2005): 1538–1551.
- Mattana, E., Pritchard, H. W., Porceddu, M., Stuppy, W. H., Bacchetta, G. “Interchangeable effects of gibberellic acid and temperature on embryo growth, seed germination and epicotyl emergence in *Ribes multiflorum* ssp. *sandaliticum* (Grossulariaceae).” *Plant Biology* 14 (2012): 77–87.
- McDonald. “Seed priming. ” In *Seed technology and its biological basis.*, by M., Bewley J. D. Black, 287–325. Sheffield: Sheffield Academic Press, 2000.
- McDonald, M. B., Kwong, F. Y. *Flower Seeds: Biology and Technology*. Cambridge: CABI Publishing, 2005.
- McDonough, W. T. “Dormant and non-dormant seeds: similar germination responses when osmotically inhibited.” *Nature* 214 (1967): 1147–1148.
- McDonough, W. T. “Germination polymorphism in *Grindelia squarrosa*.” *Northwest Science* 49 (1975): 190–200.
- McEvoy, P.B. “Dormancy and dispersal in dimorphic achenes of tansy ragwort, *Senecio jacobaea* L. (Compositae).” *Oecologia* 61 (1984): 160–168.
- McQueen-Mason S., Fry, S. C., Durachko, D. M., Cosgrove D. J. “The relationship between xyloglucan endotransglycosylase and in vitro cell wall extension in cucumber hypocotyls.” *Planta* 190 (1993): 327–331.
- McQueen-Mason, S., D. J. Cosgrove. “Expansin mode of action on cell walls. Analysis of wall hydrolysis, stress relaxation, and binding.” *Plant Physiologist* 107 (1995): 87–100.
- Meinke, D. W., Cherry, J. M., Dean, C., Rounsley, S. D., Koornneef, M. “*Arabidopsis thaliana*: a model plant for genome analysis.” *Science* 282, no. 5389 (1998): 679–682.
- Meusel, H., E. Jäger, E. Weinert. *Comparative Chorology of the Central European Flora*. Jena: Gustav Fischer Verlag, 1965.

- Mukherjee, S. K., Sarkar A. K. “Cypselar morphology and anatomy in some members of the family Compositae – tribe Anthemideae.” In *Proceedings of the national symposium of plant sciences in the nineties*, by R. D., Sen S. P., Samaddar KR, Sen U, Sarkar AK and Biswas AK Banerjee, 448-464. Kalyani: Department of Botany, Kalyani University, 1992.
- Mullen, R. T., J. E. King, D. J. Gifford. “Changes in mRNA populations during loblolly pine (*Pinus taeda*) seed stratification, germination and postgerminative growth.” *Physiologia Plantarum* 97 (1996): 545-553.
- Nakabayashi, K., Bartsch, M., Xiang, Y., Miatton, E., Pellengahr, S., Yano, R., Seo, M., Soppe, W, J. “The time required for dormancy release in *Arabidopsis* is determined by DELAY OF GERMINATION1 protein levels in freshly harvested seeds.” *The Plant Cell* 24, no. 7 (2012): 2826-2838.
- Nakabayashi, K., Okamoto, M., Koshiba, T., Kamiya, Y., Nambara, E. “Genome-wide profiling of stored mRNA in *Arabidopsis thaliana* seed germination: epigenetic and genetic regulation of transcription in seed.” *The Plant Journal* 41, no. 5 (2005): 697-709.
- Nascimento, W. M., West S. H. “Priming and seed orientation affect emergence and seed coat adherence and seedling development of muskmelon transplants.” *Horticultural Science* 33 (1998): 847–848.
- Naylor, R. E. L. *Weed Management Handbook*. John Wiley & Sons, 2008.
- Negbi M., Tamari B. “Germination of chlorophyllous and achlorophyllous seeds of *Salsola volkensii* and *Aellenia autrani*.” *Israel Journal of Botany* 12 (1963): 124–135.
- Nikolaeva, M.G. *Physiology of deep dormancy in seeds*. Israel Program for Scientific Translations, 1969.
- Nirmala A., Rao P. N. “Somatic chromosome morphology in Asteraceae.” *Cell and Chromosome Research* 9 (1986): 26-27.
- Olmstead, R. G., Michaels, H. J., Scott, K. M., & Palmer, J. D. “Monophyly of the Asteridae and identification of their major lineages inferred from DNA sequences of *rbcl*.” *Ann. Mo. Bot. Gard.* 79 (1992): 249-265.
- Osborne, D. J., L. L. Boubriak. “DNA and desiccation tolerance.” *Seed Science Research* 4 (1994): 175-185.
- Parera, C. A., Cantliffe D. J. “Improved germination and modified imbibitions of shrunken-2 sweet corn by seed disinfection and solid matrix priming.” *Journal of American Society Horticultural Science* 116 (1991): 942–945.
- Pavone, P., Terrasi, C.M., Zizza, A. “Chromosome number reports LXXII.” *Taxon* 30 (1981): 695-696.
- Peeters, A. J., Blankestijn-De Vries, H., Hanhart, C. J., Léon-Kloosterziel, K. M., Zeevaart, J. A., Koornneef, M. “Characterization of mutants with reduced seed dormancy at two novel *rdo* loci and a further characterization of *rdo1* and *rdo2* in *Arabidopsis*.” *Physiologia Plantarum* 115, no. 4 (2002): 604-612.
- Pemadasa, M.A., Lovell, P.H. “Factors controlling germination of some dune annuals.” *Journal of Ecology* 63 (1975): 41–59.
- Pennisi, E. “Wanted: A Barcode for Plants.” *Science* 318, no. 5848 (2007): 190-191.
- Pignatti, S. *Flora d'Italia*. Bologna: Edagricole, 1982.
- Pritchard, J., Hetherington, P. R., Fry, S. C., Tomos, A. D. “Xyloglucan endotransglycosylase activity, microfibril orientation and the profiles of cell wall properties along growing regions of maize roots.” *Journal of Experimental Botany* 44 (1993): 1281-1289.

- Probert, R.J. “The Role of Temperature in the Regulation of Seed Dormancy and Germination.” In *Seeds: the ecology of regeneration in plant communities.*, by M. Fenner, 261-292. CABI, 2000.
- Qian, W., Ge S., Hong D. Y. “Genetic variation within and among populations of a wild rice *Oryza granulata* from China detected by RAPD and ISSR markers.” *Theoretical Applied Genetics* 102 (2001): 440–449.
- Rajjou, L., K. Gallardo, I. Debeaujon, J. Vandekerckhove, C. Job, D. Job. “The effect of alpha-amanitin on the Arabidopsis seed proteome highlights the distinct roles of stored and neosynthesized mRNAs during germination.” *Plant Physiology* 134 (2004): 1598–1613.
- Ratnasingham, S. and Hebert P. D. N. “BOLD: The Barcode of Life Data System.” *Molecular Ecology Notes* 7, no. 3 (2007): 355–364.
- Razaq Z.A., Khatoon S., Ali S.I. “A contribution to the chromosome numbers of Compositae from Pakistan.” *Pakistan Journal of Botany* 20 (1988): 177-189.
- Rocha, O.J. “The effects of achene heteromorphism on the dispersal capacity of *Bidens pilosa*.” *International Journal of Plant Sciences* 157 (1996): 316–322.
- Rodríguez, M. V., P. E. Toorop, and R. L. Benech-Arnold. *Challenges Facing Seed Banks and Agriculture in Relation to Seed Quality*. Vol. 773, in *Seed Dormancy, Methods in Molecular Biology*, by Kermode A. R., 17-40. Humana Press, 2011.
- Rouhi, H. R., Aboutalebian M. A., Sharif-Zadeh F. “Effects of hydro and osmopriming on drought stress tolerance during germination in four grass species.” *International Journal Agricultural Science* 1 (2011): 704-774.
- Rubinoff, D., Cameron S., Will. K. “A Genomic Perspective on the Shortcomings of Mitochondrial DNA for "Barcoding" Identification.” *Journal of Heredity* 97, no. 6 (2006): 581-594.
- Sánchez-Martínez, D., P. Puigdomènech, M. Pagès. “Regulation of gene expression in developing Zea mays embryos. Protein synthesis during embryogenesis and early germination of maize.” *Plant Physiology* 82 (1986): 543-549.
- Sanchez, R. A., Mella, R. A. “The exit from dormancy and the induction of germination: Physiological and molecular aspects.” In *Handbook of seed physiology: applications to agriculture.*, by R. L., Sanchez, R. A. Benech-Arnold, 221–243. New York: Food Product Press and the Haworth Reference Press, 2004.
- Santamaría, M. E., Rodríguez, R., Cañal, M. J., Toorop, P. E. “Transcriptome analysis of chestnut (*Castanea sativa*) tree buds suggests a putative role for epigenetic control of bud dormancy.” *Annals of Botany* 108, no. 3 (2011): 485-498.
- Schaefer, H., Hardy, O.J., Silva, L., Barraclough, T.G., Savolainen, V. *Ecology Letters* 14 (2011): 389–396.
- Scheibe, J., A. Lang. “Lettuce seed germination: Evidence for a reversible light induced increase in growth potential and for phytochrome mediation of low temperature effect.” *Plant Physiology* 40 (1965): 485-492.
- Schmid, M., T. S. Davison, S. R. Henz, U. J. Pape, M. Demar, M. Vingron, B. Schölkopf, D. Weigel, J. U. Lohmann. “A gene expression map of Arabidopsis thaliana development.” *Nat Genet.* 37, no. 5 (2005): 501-506.
- Schmittgen, T.D., Zakrajsek B.A. “Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR.” *J Biochem Biophys Methods* 46, no. 1-2 (2000): 69-81.
- Schopfer, P., Placy, C. “Control of seed germination by abscisic acid. Effect of embryo growth potential (minimum turgor pressure) and growth coefficient (cell wall extensibility) in *Brassica napus* L.” *Plant Physiology* 77 (1985): 676-686.

- Shen, Z. X., Parrish, D. J., Wolf, D. D., and Welbaum, G. E. “Stratification in Switchgrass Seeds Is Reversed and Hastened by Drying.” *Crop Science* 41 (2001): 1546–1551.
- Shih, C., Fu G. *Angiospermae Dicotyledoneae Compositae 3 Anthemideae*. Peking: Science Press, 1983.
- Smissen, R. D., I. Breitwieser, J. M. Ward, P. A. McLenachan, and P. J. Lockhart. “Use of ISSR profiles and ITS-sequences to study the biogeography of alpine cushion plants in the genus *Raoulia* (Asteraceae).” *Plant Syst. Evol* 239 (2003): 79–94.
- Strother, J. L., L. E. Watson. *Chromosome numbers in Compositae from Morocco and Spain*. Vol. 17, in *Documented chromosome numbers*, by Watson L.E. Strother J.L., 627–629. Sida, 1997.
- Tanowitz, B. D., Salopek P. F., Mahall B. E. “Differential germination of ray and disc achenes in *Hemizonia increscens* (Asteraceae).” *American Journal of Botany* 74 (1987): 303–312.
- Taylor, A. G., Harman G. E. “Concepts and technologies of selected seed treatments.” *Annals Review Phytopathology* 28 (1990): 321–329.
- Thomas, U. C., Varughese K., Thomas A., Sadanandan S. *Seed priming for increased vigour, viability and productivity of upland rice*. Vol. 4. 14 vols. Leisa , 2000.
- Thompson, K., Rabinowitz D. “Do Big Plants Have Big Seeds?” *The American Naturalist* (The University of Chicago Press) 133, no. 5 (1989): 722–728.
- Thompson, P. A. “Changes in germination responses of *Silene secundiflora* in relation to the climate of its habitat.” *Physiologia Plantarum* 23 (1970): 739–746.
- Toorop, P. E., Aelst A. C., Hilhorst H. W. M. “The second step of the biphasic endosperm cap weakening that mediates tomato (*Lycopersicon esculentum*) seed germination is under control of ABA.” *Journal of Experimental Botany* 51, no. 349 (2000): 1371–1379.
- Toorop, P. E., Barocco R. M., Engler G., Groot S. P. C., Hilhorst H. V. M. “Differentially expressed genes associated with dormancy or germination of *Arabidopsis thaliana* seeds.” *Planta* 221 (2005): 637–647.
- Turland, N.J. “Proposal to conserve the name *Chrysanthemum coronarium* (Compositae) with a conserved type.” *Taxon* 53, no. 4 (2004): 1072–1074.
- Van Staden, J., Manning, J. C., Kelly, K. M. “Legume seeds - the structure: function equation.” In *Advances in Legume Research*, 417–450. St Luis: Stirton, C. H., Zarucchi, J. I., 1989.
- Venable, D. L. “Bet hedging in a guild of desert annuals.” *Ecology* 88 (2007): 1086–1090.
- Vicente, M.J., Martínez Sánchez, J.J., Conesa, E., Cros, V., Franco, J.A. “Seed germination studies on *Chrysanthemum coronarium*.” *VII International Workshop on Seed Biology*. Salamanca, 2002.
- Vleeshouwers, L. M., Bouwmeester H. J., Karssen C. M. “Redefining seed dormancy: an attempt to integrate physiology and ecology.” *Journal of Ecology* 83 (1995): 1031–1037.
- Vogt R., Oberprieler C. “Mediterranean chromosome number reports.” *Flora Mediterranea*, 1993: 338–340.
- Wagmann, K., Hautekèete, N. C., Piquot, Y., Meunier, C., Schmitt, S. E., Van Dijk, H. “Seed dormancy distribution: explanatory ecological factors.” *Annals of Botany* 110 (2012): 1205–1219.

-
- Wan, C-Y, T. A. Wilkins. “A modified hot borate method significantly enhance the yield of high quality RNA from cotton (*Gossypium hirsutum* L.).” *Analytical Biochemistry* 223 (1994): 7-12.
 - Watkins, J. T., D. J. Cantliffe. “Mechanical resistance of the seed coat and endosperm during germination of *Capsicum annuum* at low temperature.” *Plant Physiology* 72 (1983): 146-150.
 - Will, K. W., Mishler B. D., Wheeler Q.D. “The perils of DNA barcoding and the need for integrative taxonomy.” *Systematic Biology* 54, no. 5 (2005): 844-851.
 - Williams, J. G. K., Kubelik A. R., Livak K. J., Rafalski J.A., Tingey S.V. “DNA polymorphisms amplified by arbitrary primers are useful as genetic markers.” *Nucleic Acid Research* 18 (1990): 6531-6535.
 - Winter, D., Vinegar B., H. Nahal, R. Ammar, G. V. Wilson, N. J. Provart. “An “Electronic Fluorescent Pictograph” Browser for Exploring and Analyzing Large-Scale Biological Data Sets.” *Plos One* 2, no. 8 (2007): e718.
 - Wolff, K., Van Rijn J.P., Hofstra H. “RFLP analysis in chrysanthemum probe and primer development.” *Theoretical Applied Genetics* 88 (1994): 472-478.
 - Wu, Y., W. G. Spollen, R. E. Sharp, P. R. Hetherington, S. C. Fry. “Root growth maintenance at low water potentials. Increased activity of xyloglucan endotransglycosylase and its possible regulation by abscisic acid.” *Plant Physiology* 106 (1994): 607-615.
 - Yagmur M., Kaydan D. “Alleviation of osmotic strength of water and salt in germination and seedling growth of triticale with seed priming treatments.” *African Journal of Biotechnology* 7 (2008): 2156–2162.
 - Zietkiewicz E., Rafalski A., Labuda D. “Genome fingerprinting by simple sequence repeat (SSR) – anchored polymerase chain reaction amplification.” *Genomics* 20 (1994): :176–183.
 - Zlatanova, J. S., P. V. Ivanov, L. M. Stoilov, K. V. Chimshirova, B. S. Stanchev. “DNA repair precedes replicative synthesis during early germination of maize.” *Plant Molecular Biology* 10 (1987): 139-144.

