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# An *in vitro* bioassay for the evaluation of cold treatment on flower bud dormancy in *Camellia*

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## Abstract

***Camellia japonica*** **L. is an evergreen shrub whose cultivars are of great ornamental value. Initiation and early differentiation of flower buds in *C. japonica* starts from late spring on while flower bud development and visible bud enlargement sequel until autumn. In many temperate woody ornamentals dormancy is installed after flower bud differentiation. The exposure of floral buds to cold temperatures (between 2-7°C) is supposed to stimulate the initiation of normal growth and anthesis during the next spring. As an attempt to quicken the fulfillment of the cold need, as well as to homogenize flowering, five low temperature regimes, consisting of darkness and a constant temperature of 7°C applied for 0, 2, 4, 6 and 8 weeks, were tested on budded plants of cultivar 'Nuccio's Pearl'. As indicator of dormancy release, an inexpensive and easygoing test was conducted by forcing excised flower buds with an *in vitro* bioassay. To infer about the reliability of this test, a possible hormonal basis was assessed by measuring the concentration of**

**abscisic acid in floral buds. Four groups of camellias of ten specimens each, treated respectively for 0, 4, 6 and 8 weeks, were then forced in the greenhouse with supplementary lighting and semi-controlled temperature for qualitative and quantitative evaluations of flowering. Our results indicated that the bioassay is a suitable indicator of the moment of dormancy release as it was able to highlight a reduced amount of dormancy when buds stayed longer at cold. Abscisic acid content in floral buds showed to be reduced and homogenized after 6 and 8 weeks of cold, thereby promoting a more uniform flowering. Hence, the bioassay appears as a cost-effective tool that could be of interest for breeders, to characterize the chilling requirements of flowers of parental plants or their offspring.**

## INTRODUCTION

*Camellia japonica* L. (Theales, Theaceae) is an acidophilic evergreen shrub whose 600 known varieties (Mondal et al., 2002) are of great ornamental value. Such species represents commercially one of the showpieces of the Lake Maggiore (Piedmont, Italy) production, with more than 200 traded cultivars characterized by a breathtaking flowering.

The qualitative and quantitative maximization of ornamental features related to flowering has recently become a most desired target among camellia growers. On top, flowers production, flowering earliness and uniformity appear to be interesting parameters which require to be controlled for the correct achievement of a marketable pot production. In this context, hastening flower bud dormancy could remarkably affect most of the features related to flower characteristics. Initiation and early differentiation of flower buds in *C. japonica* start in late spring while flower bud development and visible

bud enlargement sequel until autumn. In many temperate woody ornamental plants dormancy is installed after flower bud differentiation. The exposure of floral buds to cold temperatures (between 2-7°C) is supposed to stimulate the initiation of normal growth and anthesis during the next spring. The length of the cold treatment needed for the resumption of growth is defined as the chilling requirement. This role of vernalization has been confirmed in model plants, such as *Arabidopsis* (Burn et al., 1993; Sheldon et al., 2000), as well as in genera phylogenetically closer to *Camellia*, such as *Rhododendron* (Criley, 1985). Hormonal bases could be represented by abscisic acid (ABA) whose concentration was reported to be high in deeply dormant vegetative buds and then reduced during dormancy release (Nagar, 1996) in *C. sinensis*, i.e. the tea plant. Interestingly, Pemberton et al. (1985) have demonstrated that a similar trend was followed by ABA content in dormant flower buds of azalea. This was later confirmed by Christiaens et al. (2011).

In this study we introduce the use of cold treatment and forcing in *C. japonica* cultivation cycle, with the purpose of assessing any effects on flowering and of defining the chilling requirement that best fits this ornamental plant. The quantification of flower buds ABA content and a set of morphological surveys were used to describe plants behavior during and after the cold treatments. Similarly to Christiaens et al. (2011), so as to select a suitable and reliable indicator of dormancy release, an inexpensive and easygoing test was conducted by forcing excised flower buds and monitoring their subsequent dormancy release during an in vitro bioassay.

## MATERIALS AND METHODS

### Plant Material

In February 2009, three rooted cuttings of *C. japonica* 'Nuccio's Pearl' were potted into peat-based standard substrate. After re-potting, plants were sprayed twice with Flurprimidol in April 2010, so as to promote flower bud initiation and differentiation (Larcher et al., 2011). Next autumn, when floral primordia were fully developed increasing cold treatments (7°C from 0 till 8 weeks) to quicken the fulfillment of flower bud dormancy requirements were applied. Subsequently, plants were forced to flower with semi-controlled temperature (night min 5 °C – day min 18 °C) and supplementary lighting.

## In vitro bioassay

At weeks 0, 2, 4, 6, and 8 flower buds were harvested from plants at 7°C. These flower buds were sterilized with 70% ethanol, 1% mercury (I) chloride, 10% sodium hypochlorite and 4% PPM (Plant Preservative Mixture – Plant Cell Technology Inc., Washington, DC, USA). The outer bud scale was removed and buds were transferred onto solid medium (7 g/l agar) in test tubes. The McCown Woody Plant Medium including vitamins (Duchefa Biochemie bv) was used. Bud dormancy release was considered to occur when the scales unfurled, opened or swelled. Every 2-3 days the number of buds that had overcome dormancy was counted, for a maximum of 108 days. Twenty four buds were used per treatment and after 12 weeks the mean values were calculated. The earliness of dormancy release as days between the start of the bioassay and 25% of buds breaking dormancy was also taken into account.

## Determination of endogenous ABA

Extraction and purification was done as described by Chen et al. (1997). Grounded samples were extracted in cold 80% aqueous methanol (5ml/g FW) overnight at 4°C with

ca 10 mg/l butylated hydroxytoluene to prevent oxidation. After centrifugation at 10000g (4°C, 20 min), the supernatant was filtered through a C18 Sep-Pak cartridge (Waters). The efflux was collected and dried in a stream of $N_2$. The residue was dissolved in 1.5 ml phosphate-buffered saline (0.01 M, pH 9.2) and adjusted to pH 8.5, then partitioned three times with an equal volume of ethyl acetate. The remaining water phase was adjusted to pH 2.5 and again extracted three times with ethyl acetate. The ethyl acetate phases were pooled and dried in a stream of $N_2$. The residue was dissolved in 200 µl of 100% methanol and diluted with tris-buffered saline (25 mM, pH7.5). ABA levels were quantified by ELISA with a phytodetek-ABA kit (Agdia, Biofords).

## Assessment of flowering quality and growth

Flowering was followed on four groups of camellias of ten specimens each, treated respectively for 0, 4, 6 and 8 weeks. For a qualitative and quantitative evaluation of flowering and growth, a set of surveys was carried out during the entire forcing phase and included height and percentage of sprouting vegetative buds, flower buds size, number of flowers produced, vase-life and flower sizes. After 20 weeks from the beginning of the experimentation, flower petals color was assessed too, according to Adamse et al. (1989) with minor modifications, using cyanidin chloride as standard. The uniformity of flowering as days between 0 and 100% flowering and the flowering earliness as days between start of forcing and 25% flowering were also determined.

# RESULTS AND DISCUSSION

## Dormancy release and flowering quality

The earliness of buds opening during the bioassay and the mean days before bud

dormancy release showed not to be correlated with the length of the cold treatment, in accordance with other studies (Barros and Neill, 1986; Christiaens et al., 2011). Anyway, interesting values were obtained with buds put in culture after 8 weeks of cold treatment. Here, the mean number of days needed for buds to release dormancy was strongly reduced while their earliness showed to be enhanced (Fig. 1).

The concentration of ABA in flower buds resulted very heterogeneous, according to the wide ranged standard error reported for the biological replicates after 0, 2 and 4 weeks at cold, and a general decreasing trend was seen during the cold treatment (Fig. 2). These data are quite in line with what reported on Table 1 about flowering characteristics, as the most uniform flowering was seen on plants treated 6 and 8 weeks with cold, possibly due to the fact that ABA content in flower buds was lower and more homogeneous too (Fig. 2). Surveys on flowering characteristics also indicated that longer cold treatments promoted an earlier and enhanced flower production (Tab. 1). Moreover, the ratio between flowers that opened and buds that started the opening process (Anthesis completion ratio) was visibly the highest when ABA content was low in buds, after 8 weeks of cold. Interestingly, the concentration of ABA was linearly correlated with the earliness of flowering while no correlation was seen with the earliness calculated on the bioassay (data not shown). Anyway, the highest earliness registered during the bioassay corresponded to the lowest value of ABA content, which corresponded also to the lowest value of mean days needed to release dormancy for buds placed in vitro. Therefore, the earliest flowering corresponded to the earliest bioassay, suggesting that the bioassay was a reliable indicator of the fulfillment of the chilling requirement.

Flowers ornamental characteristics are shown in Table 2. The mean volume of flowers decreased with longer cold treatments, unfortunately, along with vase life. Also,

petals cyanidin chloride content showed to be reduced after a cold treatment longer than 4 weeks, causing flowers to turn out less pinkish. These negative side effects could be due to the fact that dormancy was also broken in vegetative buds (Tab. 2), on which longer cold treatments promoted an active sprouting soon before the end of flowering. This overlap with flowering process was probably too early, causing too many sinks to be in need of plant support at the same moment.

**Conclusions**

In conclusion, flowering was affected by the amount of cold received during dormancy. Generally, phenological effects could be resumed by an increased, earlier, more homogeneous and less pinkish flowering. Some negative side effects on vase life and size of flowers were seen too. Future studies should concentrate on how to overcome the negative side effects seen on flowering.

The bioassay, an easygoing, low input and inexpensive test showed to be a good indicator of the moment of dormancy release which suggested, as confirmed by the ABA levels assessment, that the chilling requirement of *Camellia japonica* 'Nuccio's Pearl' could be defined as 8 weeks at 7 °C with no light supply. This cost-effective tool could, therefore, be of particular interest for breeders willing to characterize the chilling requirements of flowers of parental plants or their offspring.

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**Tables**

Table 1: Effects of cold treatment on flowering quantitative parameters

| Weeks at cold + dark | N. of flowers | Anthesis completion ratio | Uniformity (days) | Earliness (days) |
|---|---|---|---|---|
| 0 | 21 | 0.53 | 80 | 114 |
| 4 | 23 | 0.70 | 73 | 93 |
| 6 | 23 | 0.85 | 70 | 72 |
| 8 | 29 | 0.94 | 70 | 57 |

Table 2: Effect of cold treatment on flowers ornamental characteristics and vegetative growth

| Weeks at cold + dark | Vase life (days) | Flower volume ($cm^3$) | Cyanidin chloride levels (µmol/g FW) | Sprouting vegetative buds (%) |
|---|---|---|---|---|
| 0 | 24.14b[1] | 106.75c | 0.240b | 0.00a |
| 4 | 15.74a | 91.07b | 0.256b | 1.25a |
| 6 | 14.78a | 72.49a | 0.195a | 42.14b |
| 8 | 14.14a | 67.88a | 0.174a | 64.54b |

[1]Means followed by the same letter do not differ significantly at $P<0.05$, according to REGW-F test for parametric data or the Mann-Whitney U test with Bonferroni transformation for non-parametric data

**Figures**

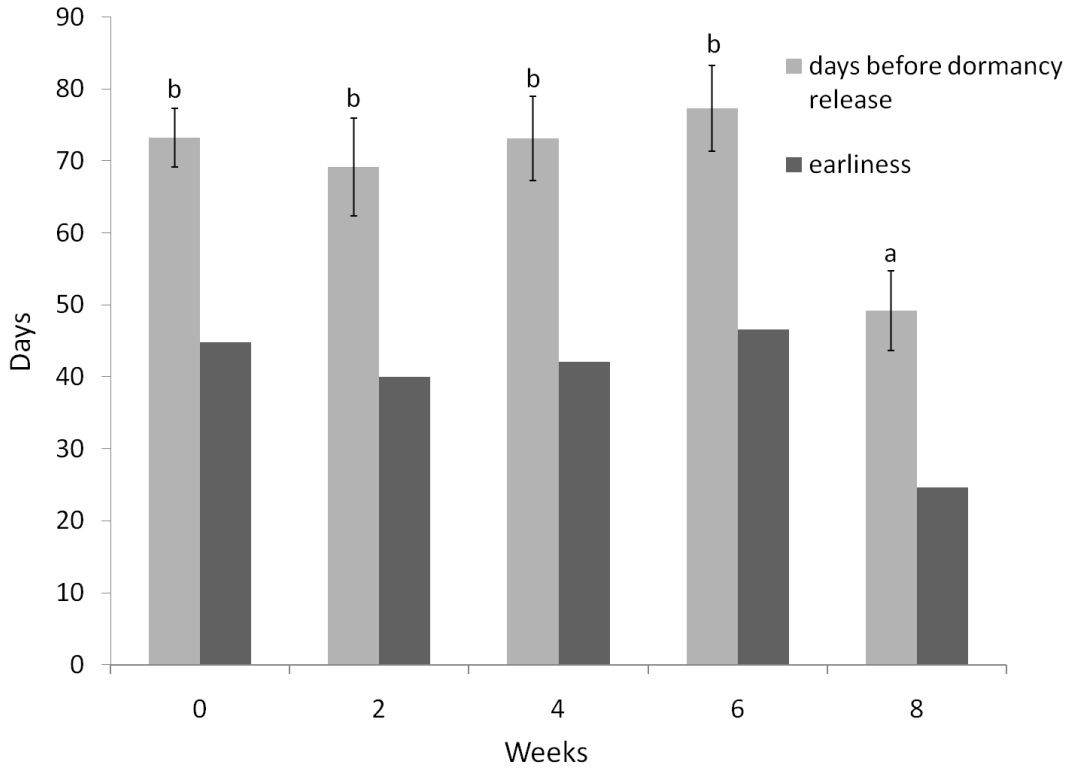


Fig. 1: *In vitro* dormancy breaking of flower buds after 0, 2, 4, 6 and 8 weeks of cold treatment. Standard Error bars are displayed for the days before dormancy release. The same letter indicates no significant differences at $P<0.05$, according to REGW-F test

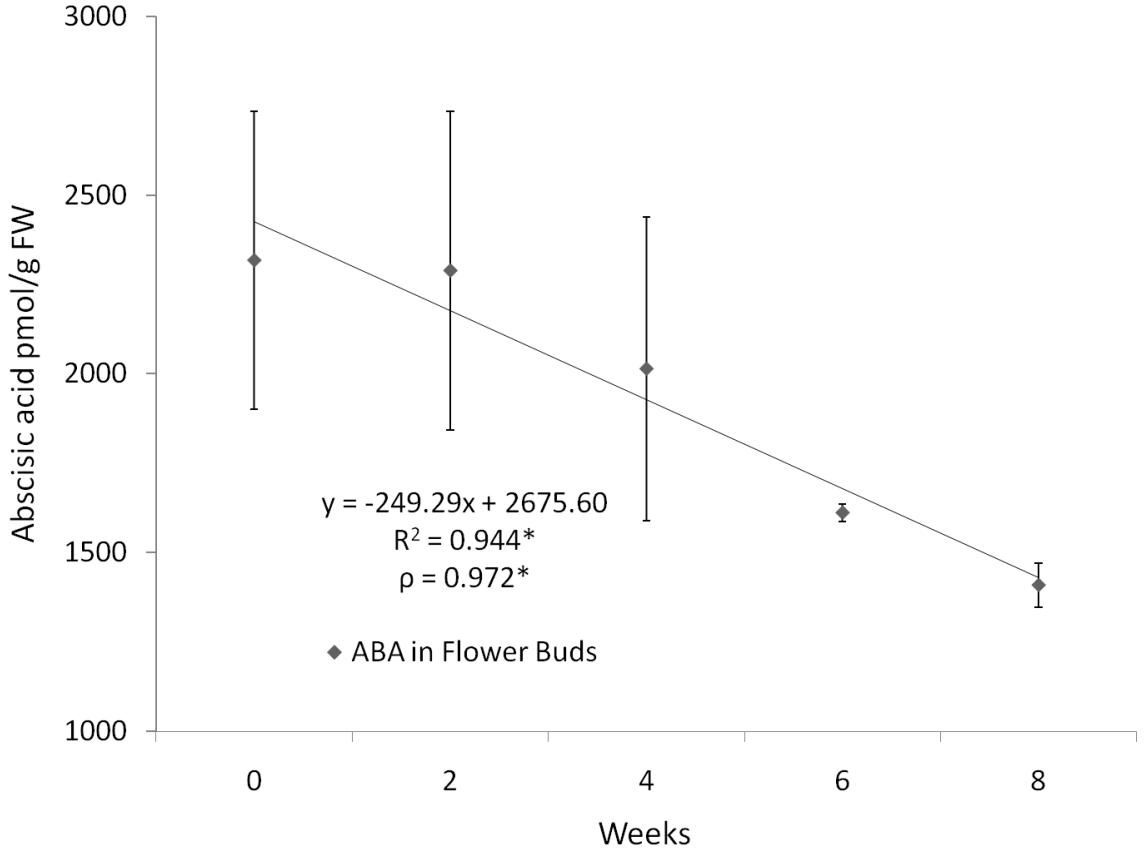


Fig. 2: Abscisic acid levels in flower buds after 0, 4, 6 and 8 weeks of cold treatment. Standard Error bars, linear regression equation, $R^2$ and Pearson's correlation coefficient (ρ) are displayed. * represents a statistical relevance of $P<0.05$