EVALUATION OF THE REPRODUCTION PROFICIENCY OF CARNATION (*Dianthus caryophyllus L.*) HYBRIDS AND VARIETIES AS SEARCH OF USEFUL PARENTALS FOR A BREEDING PROGRAM

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EVALUACION DE LA CAPACIDAD REPRODUCTIVA DE HÍBRIDOS Y VARIEDADES DE CLAVEL (*Dianthus caryophyllus L.*) COMO BUSQUEDA DE PARENTALES ÚTILES DENTRO DE PROGRAMAS DE FITOMEJORAMIENTO

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ABSTRACT

Colombia is the world's leading exporter of carnation (*Dianthus caryophyllus L.*) and the second of fresh cut flowers, that is why the floriculture is one of the economic sectors that generate more exchange in exports of our country. However, carnation growers must face the problem of decreasing revenues, due to the rot vascular disease produced by the pathogen *Fusarium oxysporum* f.sp. dianthi. Studies show that the most effective way to face the problem is to find resistant carnation varieties, obtained by breeding programs. Thus, here we present a floral evaluation of the gynoecium and androecium, possible flower defects and the reproduction capacity as well, all this useful for the breeding of four carnation commercial varieties and two hybrid lines, all of them obtained through a breeding program focus on *Fusarium* sp. resistance. Direct and reciprocal breeding crosses were done between all materials in order to establish in which crosses were produced viable seeds of F1 populations. As a result, the reproduction capacity of the six parental was established and a total of 809 seeds were produced from nine successful crosses. Also, it was established that floral opening stage II, is the stage in which the pollen is mature and viable. Moreover, it was established that Kaly, Candy and UM503 were the parental that have best performances as acceptor; in contrast UM226 was the best pollen donator. On the other hand, Lady Green had poor performance both as donor and acceptor.

Key words: pollination, gynoecium, androecium

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RESUMEN

Colombia es el primer exportador mundial de clavel (*Dianthus caryophyllus L.*) y el segundo de flor fresca cortada, haciendo de la floricultura uno de los sectores que genera más divisas en las exportaciones de nuestro país. Sin embargo, los productores de clavel están enfrentados al problema de la disminución en ganancias y en área cultivada de clavel, debido a la marchitez vascular producida por el patógeno *Fusarium oxysporum* f.sp. dianthi. Los resultados de numerosos estudios muestran que la manera más efectiva de tratar el problema es la búsqueda de variedades resistentes utilizando métodos tradicionales de fitomejoramiento. Esta investigación tuvo como objetivos, realizar una evaluación floral del gineceo y androceo y establecer la capacidad reproductiva útil para la hibridación, de cuatro variedades comerciales y dos líneas híbridas de clavel, producto del programa de fitomejoramiento de la Universidad Militar Nueva Granada, así como la realización de cruces, por hibridación varietal, directa y recíproca entre el material evaluado, para establecer en cuales cruces se presenta producción de semilla viable y obtener así, poblaciones F1. Como resultados, la capacidad reproductiva de seis parentales fue establecida y se obtuvieron nueve cruces exitosos (poblaciones), entre ellos, con un total de 809 semillas híbridas. Se estableció que el estadio de apertura floral II es aquel en donde se encuentra polen maduro y con mayor porcentaje de viabilidad. Además se determinó que en la hibridación, los parentales Kaly, Candy y UM503 fueron los que tuvieron mejor comportamiento como receptores y que el parental UM226, fue el mejor donador. La variedad Lady Green tuvo un comportamiento deficiente tanto como donante como receptor en la obtención de semilla viable de clavel.

Palabras claves: polinización, androceo, gineceo

INTRODUCTION

Colombia is the carnation´s first world exporter and the second producer of cut fresh flower, with a crop area of 7.200 Ha, where the carnation is the 18% of all the flower production. The principal markets are North America and the European Union. The profits have increased up to 1.050 million dollars and this industry has generated more than 170.000 employs as well.

However, the carnation producers are in profits of a problem, which is the decrease of profits and crop area, due to the vascular wilt, caused by the pathogen *Fusarium oxysporum* f.sp. dianthi. Some results of our investigations show that the most effective form to add ress the problem is to research or develop resistant varieties, using traditional breeding methods (Arbeláez, 1987; Filgueira, 2009).

The "Nueva Granada" Military University (UMNG) leads a carnation breeding program, unique in the country, where its principal objective is the search of commercial material, with resistance to *Fusarium* sp. pathogens. The development and sowing of resistant varieties entails an important reduction of costs in production and avoids losses caused by the disease. In the entire world, the carnation breeding programs are looking for new colors, more and best development, and resistance to plagues. In countries like Holland, Italy and United States, the breeding research has been focused in the development

of resistant material to devastating diseases like the vascular wilt produced by *Fusarium oxysporum* (Arus *et al.*, 1992). In fact, the varieties here evaluated are promising materials inside the breeding program, because they have contrasting resistance levels.

Historically, carnations have been reported as one of the first artificial hybrids produced, and this was accomplished by the Dr. Thomas Fairchild, with a cross between *D. caryophyllus* and *D. barbatus*, around the 1716 (Rieseberg and Carney, 1998). Carnation belong to the Caryophyllaceae family, is an allogamous plant, ornamental, hermaphrodite, hypogyny, and wit a central ovary placentation, that has been used by the man through ages (McDonald and Kwong, 2005). Also, the carnation presents the phenomena of protandry, where the male function precedes the female (Bertin and Newman, 1993).

It has been attributed to this dichogamy some benefits, as the possible advantage of avoid the mutual interference between the male and female functions in the flower (Brantjes, 1982; Lloyd and Yates, 1982; Wyatt, 1983; Lloyd and Webb, 1986; Bernhardt and Thien 1987, cited in Bertin and Newman, 1993). Also, the fact of favoring some early stages and prolonged of the pollen, at least by the case of the protandry (Bawa and Beach, 1981; Lloyd and Webb, 1986; Webb, 198l, cited in Bertin and Newman, 1993).

On the other hand, into a breeding program, it is important the evaluation of the floral characteristics and the estimation of the optimal time in order to obtain viable seeds. The aims of this project were to perform a floral evaluation of gynoecium and androecium, and establish the reproductive capacity of carnation materials for a breeding program. The second aim was to produce seeds by direct and reciprocal crosses, between the evaluated materials, to establish in which of these the production of viable seed was superior. Here we present the details and forward of a previous work done by the UMNG carnation breeding program, which are in press.

MATERIALS AND METHODS

Plant Material

As parental lines we used the commercial varieties Candy, Bagatel, Kaly and Lady Green, and the hybrid lines UM226 and UM503, product of the breeding program of the UMNG. In order to propagate the vegetal, each parental was introduced to tissue culture for propagation using ex vitro apical meristems in liquid media MS (Murashige and Skoog, 1962), 6000 lx, 26°C +/- 2 and 16 hours/light/day photoperiod. After four weeks and once we obtained the microplants, they were micro-propagated vegetatively to obtain 200 microplants of each plant of the parental. Later, the microplants were sown in field conditions, in germination trays, with sterile peat and high humidity conditions. After six weeks, the plants were placed into a greenhouse and were transplanted into pots of 1 kg capacity. As substratum we used rice husk and soil in a 1:1 proportion, the irrigation was done using sterile water with a nutritive solution of Master 30-40-30®.

Floral evaluation and reproductive capacity of parental lines and varieties

With the aim of establishing the reproductive capacity (crossability) and the optimal floral conditions for the pollination of commercial varieties and carnation lines, we studied the gynoecium and androecium. To gynoecium level we counted the number of ovules by flower in each parental, with five repetitions, in floral aperture II, in which the petals have exceed the half of the calyx total diameter (Fig. 1).

We evaluated the time when the stigma became receptive. For this test we took five flowers by parental at an opening stage II, and those were emasculated in T=0. Each 24 hours and during 120 hours (T1 =24 h, T2 =48 h, T3 =72 h, T4 =96 h, T5 =120 h) the stigma and papillary cells were observed to determinate the moment when the stigma is become receptive. This moment can be recognized by the

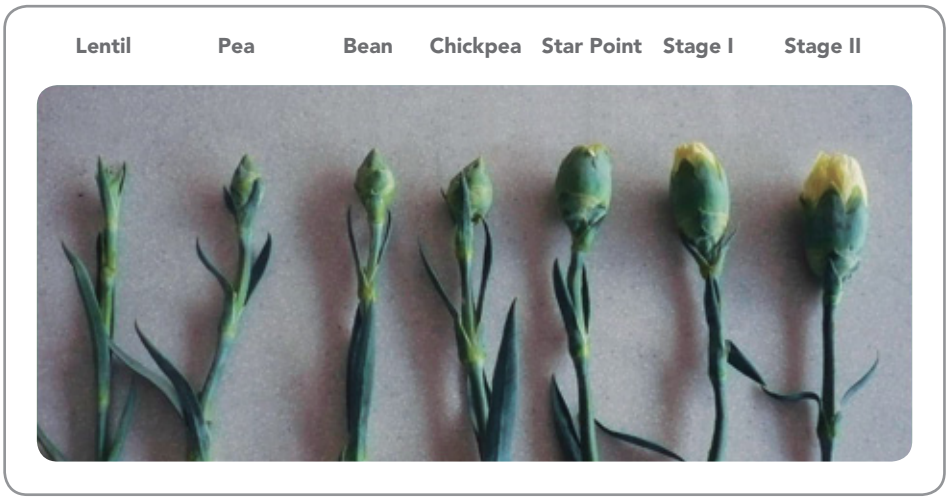

Figure 1. Floral opening stages in carnation, commercial variety Candy.

curvature of the stigma, indicating the appropriated moment to perform the pollination.

The evaluation of androecium was done through the count the pollen grains by anther, with five repetitions, taking the anther content by flower in opening stage II, then the content was suspended in 1ml of distilled water and then the counting was done using a Neubauer chamber (Soto and Filgueira, in press, Dudash, 1991; Shore and Barret, 1984; Barret, 1985 en Kears and Inouye, 1993). Also, we did observations of pollen grains when the flower was in chickpea, star, and I, II, II stages (Fig. 1), this with the purpose of determinate the floral stage when the pollen grains are mature. For this evaluation, we did Fuschine staining of pollen grains of each parental line. The test of pollen viability was done by measuring *in vitro* pollen tube germination percentage of pollen collected from flowers on stage I, II, and III, according the method of Cuellar and collaborators (1999) and Soto and Filgueira (in press).

In order to obtain viable seeds, direct and reciprocal crossings were performed between flowers of six parental lines, for a total of 18 different crosses. Finally we analyzed the variable correlations like number, seed weight and germination percentage, using the statistic program SAS 9.00.

Obtaining of seeds and germination

By parental we used 200 plants, when the plants produced flowers, those were pollinated and to avoid the cross pollination the flowers were emasculated on stage II, this stage is recognized because the petals exceed the half of the calyx longitude (Fig. 1) then we removed the petals and covered the flowers with a veil hood, the mature anthers were collected from donating flowers and were preserved in a humid chamber at 4°C, up the moment that the flower stigma was receptive. For pollinating a pin was used to spread the pollen on the stigma, this procedure was done between the 6 a.m. and 8 a.m. looking for enviromental conditions of low temperature, not more than 25°C and a high relative humidity. In successful fecundations we obtained seeds about 60 days later. A week after the pollination, the

fecundation was evident by the change in position and dehydration of the stigma, the ovary widening and the change of color from green to brown. We register the weight of each seed and then the seeds were stored at 4°C, in foil paper with fungicide.

The seed germination was performed *ex vitro*, the seeds selected were those whose weight was equal or higher than 0.3 mg and brown or black (Soto *et al*., 2005). Also, we determined the germination percentage after two weeks of being planted.

RESULTS AND DISCUSSION

Floral evaluation and reproductive capacity of parental lines and varieties

The evaluation of the ovules number by each flower shows that Bagatel was the variety that presents the highest ovules number per flower, 166, followed by the hybrid line UM 503 with an average of 97 ovules per ovary (Fig. 2).

The importance of the gynoecium evaluation is determinate by its role in the fecundation, like the pollen deposit site and its posterior germination (Kearns and Inouye, 1993). Likewise, the stigma evaluation and its secretions provide information very useful to understand the pollen behavior regarding to the viability in the pollination process (Kearns and Inouye, 1993).

The results about the ovules number, is concordant with a study done in other species of the Dianthus, where a range between 89 and 205 was reported on hermaphrodites flowers (Jurges and Gottsberger, 2001; Jurges and Gottsberger, 2002). The ovules number by ovary could be an indirect measurement of the flower potential for seed production (Rocas and Ramírez, 2006; Kearns and Inouye, 1993), reason whereby this evaluation has high importance in processes like breeding.

Kaly and Candy present an intermediate number of ovules, 131 and 136 respectively, produced the highest number of seeds. Regarding the UM226 parental, it was the lower seeds producer according to the ovules number (Fig. 2). The last is an indication that although the ovules number is a potential estimative of seeds number, a high value of this variable is not necessary correlated with a high seeds production.

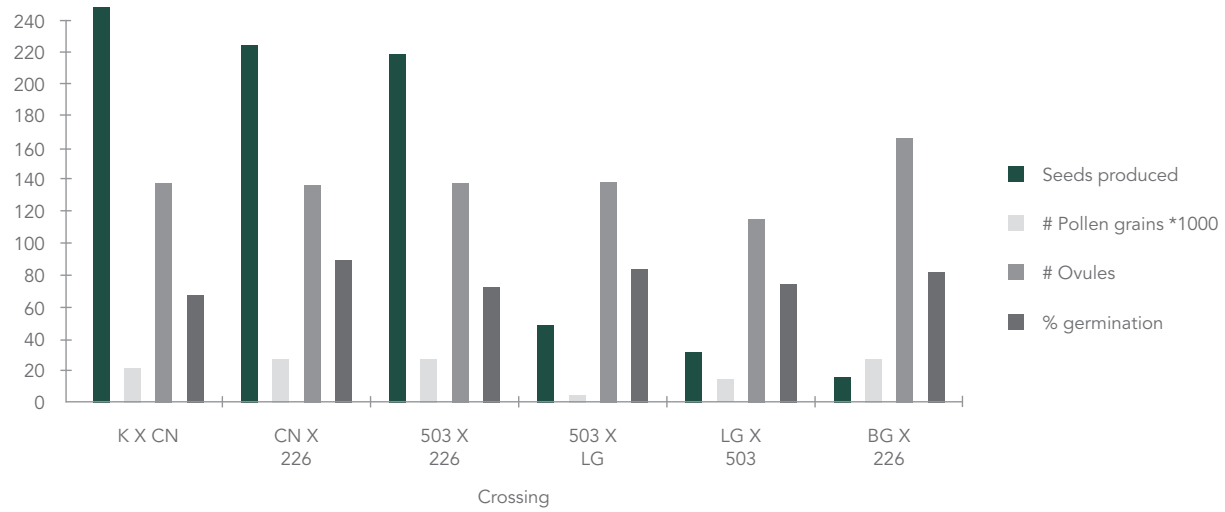

Figure. 2. Ovule number, pollen grains by parental, produced seeds, weight and germination percentage by crosses. (K=Kandy, CN=Candy, LG=Lady Green, BG=Bagatel, 226=UM226, 503=UM503, female X male).

Regarding the time evaluation of stigma receptivity, the results shows that the line UM226 was the one that presented the lowest stigma receptivity time, 24h, from the moment of emasculation, while, the UM503 line was the parental which we observed had the highest stigma receptive time, corresponding to 120 hours. However the variety Kaly, Candy and Lady Green presents receptivity times of 72 hours. The results of this evaluation show, that the time change between varieties and parental lines, depends of the variety (Fig. 3).

Figure 3. Evaluation of stigma receptivity. A. Kaly´s stigma on time 0 (emasculation), immature stigmas. B. Bagatel´s stigmas on time 4 (72 hours) receptive stigmas slightly bent.

The importance of stigma receptivity variable, lies in that the success of pollination and fecundation, is not sufficient to transfer the pollen to the stigma, but instead it is necessary to do that in the optimal moment in which the stigma is receptive, otherwise, the pollen could not adhere and not germinate (Kearns and Inouye, 1993). That fact, allow us to think that the precise time that the stigma of a variety of carnation is receptive for the fecundation, is key in a breeding program, to make the pollinating process more efficient.

There are some studies about the effect of the ethylene and the stigma receptivity in carnation (Jones, 2002; Agarwal, *et al*, 2012). However, we did not discovered in the literature, any evaluation study of receptivity time in carnation, being this article the first report on this matter (Helslop-Harrison and Shivanna, 1977; Kearns and Inouye, 1993).

An important phenomena in the floral evaluation, is the carnation dichogamy. Results obtained here do not provide sufficient evidence to award to the carnation protandry some advantages; it only was observed and takes into account in the moment to do the directed pollination.

Regarding to the androecium evaluation and specifically in the pollen count by anther, the results show that the parental that presents the highest pollen grains by anther is the line UM226, with an overage range around 27.000 pollen grains by anther, followed by the variety Candy. Moreover, the parental that presented the lowest pollen by anther was Lady Green, with an average of 4.300 pollen grains by anther. In crosses where used the parentals UM226 and Candy as donator occurred the highest production of seeds (Fig. 2). Particularly, the variety Kaly, resulted to be androsterile, does not produce pollen. Their anthers present a dry appearance, even in an initial development stage, reason why was not possible to be used as pollen donor for the crosses

There are several reasons why in the breeding programs it is useful to have the pollen count. Because with this information we can estimate the male reproductive performance as well as make comparisons between different kinds or varieties of the same species (Kears and Inouye, 1993; Dalkiliç and Mestav, 2011). Being, the two previous reasons, why this variable was taken into account.

In the microscopic observations of pollen grains stained with fuschine, the results showed that for the parental Lady Green, Bagatel, UM226 and UM503, there are mature pollen from the floral stage I, while for the case of the commercial variety Candy, the mature pollen is observed from floral stage II (Fig. 4). This result indicate that for the process of pollination,

is convenient to take the flower pollen that is in the floral stage posterior to the stage I, in that stage we can guarantee that the pollen is mature and optimal to carry out the fecundation process.

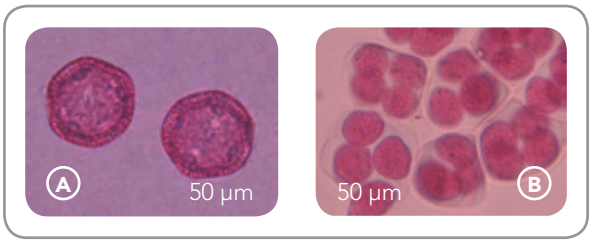

Figure 4. Carnation pollen. A. pollen of the Candy in floral chickpea stage, we can see microspores in tetragonal tetrads, evidence of pollen immaturity. B. Mature pollen.

Our results of pollen viability shows that the parent with the highest percentage of germination was the line UM226, 23%, the pollen was extracted from flowers in stage II, followed by parental Candy and UM503. While the parents that had the lowest pollen germination percentage were the commercial varieties Bagatel and Lady Green. The floral stage, in which is present the highest percentage of pollen germination, for all the parentals was the floral stage II (Fig. 5).

Microscopic examination of pollen tubes, not only is useful to estimate pollen germination, but is also used as a determinant of the effectiveness of the fertilizations in breeding programs (Mulcahy, 1979; Dalkiliç and Mestav, 2011). The results of pollen viability, with the result of the evaluation of mature, show that the parental floral stage in which the pollen is in the best conditions is the floral stage II.

Obtaining of seeds and germination

Based on the floral evaluation, were determined the parameters that we used in the breeding experiments between the six parentals. Those parameters consist in the pollination, taking in a count the time in which each parental have its stigmas receptive and the floral stage in which each parental have mature pollen. All of this in order to increase the possibility to do the pollination process successful and obtain viable seeds.

We obtained in total 809 seeds, product of nine successful crosses (Fig. 2). For each F1 seed obtained, it was evaluated its color, weight and viability (Soto *et al.*, 2005). As for the registration of seed weight per crossing, the values ranged between 1.3 and 2.7 mg. The cross that presented the heavier

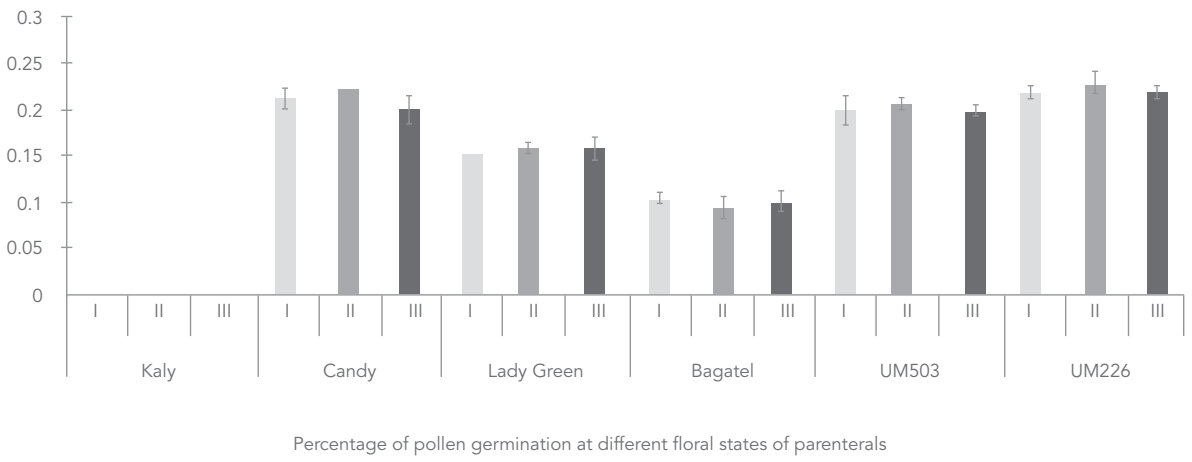

Figure 5. Germination percentage of pollen of the six parental, evaluated in the floral stages I, II and III.

seeds, corresponded to Candy X UM226, and which presents seeds with the less weight, corresponded to UM503(♀) X UN226(♂) (Fig. 2).

The seed weight parameter is vital, because the weight can be interpreted as the endosperm quantities that present the seeds, and also can be correlated to the increase or decrease of viability (Soto *et al,* 2005; Poehlman y Allen, 2003). Related to the seeds color Soto and Collaborators (2005), conclude that the dark seed coat, mainly the black, is related with the viability in the moment of seed germination, condition that was ratified and taken into account in the present work.

On the other hand, some flower defects were detected of important manner, because it affects the seed quality. The principal defect observed was the emission of secondary structures with ovary appearance, into the ovary properly. When those structures dominated "secondary ovary", are present, promoted as the condition called "cracked", producing aborts and this contribute to the opportunist parasite invasions. That floral effect in particular, was observed in the variety Lady Green (Fig. 6A).

With the aim to confirm that these structures similar to ovaries, were an ovary tissue, we performed a histological analysis of both a carnation typical ovary and a secondary structure that were generated. This analysis was developed through the technique of tissue staining

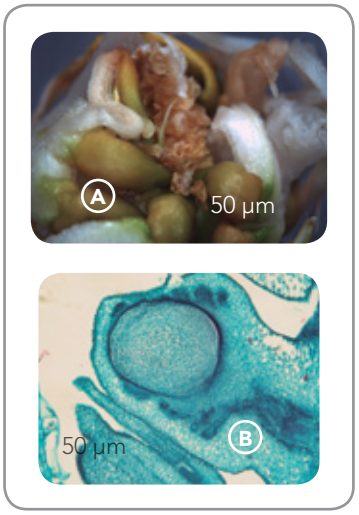

Figure 6. A. Flower of variety Lady Green with secondary ovary. B. Optic microscopy of transversal cuts of secondary ovary tissue of the variety Lady Green, we can observe immature ovary tissue and the arrows indicated mother cells of the megaspore.

Fast Green, with protocol modified by the Histoembryology laboratory at UMNG. In the histology analysis done over the "secondary ovaries", we detect immature ovary tissue and stem cells of the megaspore as well (Fig. 6B). However, there is no evidence that this tissue may play ovarian function within the floral gynoecium.

We can conclude that the opening floral II is the stage in which we can expect to find mature pollen and with high percentage of viability

On the other hand, the test of seed germination is a useful procedure to estimate the emergence percentage in a seed batch under favorable conditions, such as the substratum media, humidity levels and temperature degrees principally (McDonald and Kwong, 2005). The obtained seeds, with brown or black color and with a weight equal o above than 0.3g were selected and germinated. After two weeks was measured the germination percentage, being the highest, the corresponding to the cross Candy X UM226, with 90%. The lowest germination percentage was present in the seeds from the cross Kaly(♀)X Candy(♂), and it was 65%.

The correlation analysis between the variables, ovules number and pollen grains with the number of produced seeds and also the weight of those seeds with the germination percentage by cross, shows that are a high correlation between the variables, seeds weight with the germination percentage. That observation is consistent with the fact that endosperm quantity in a seed is correlated with its viability in the germination moment.

We did not observed a high correlation between the produced seeds by cross and the pollen grain number by anther by flower in the male parental, neither with the ovules number in the female parental. That indicates that despite of having a good number of ovules and pollen grains in the corresponding sexual organ, not necessary that is correlated with a high seed production.

Finally, through the floral evaluation of commercial varieties and carnation lines, we can conclude that the opening floral II is the stage in which we can expect to find mature pollen and with high percentage of viability. Also we determinate that the parentals Kaly, Candy and UM503 are those with the best behavior like acceptor and that the parental UM 226 was the best donator. On other hand, the carnation variety Lady Green had a deficient behavior both as donator like acceptor in the production of seeds.

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