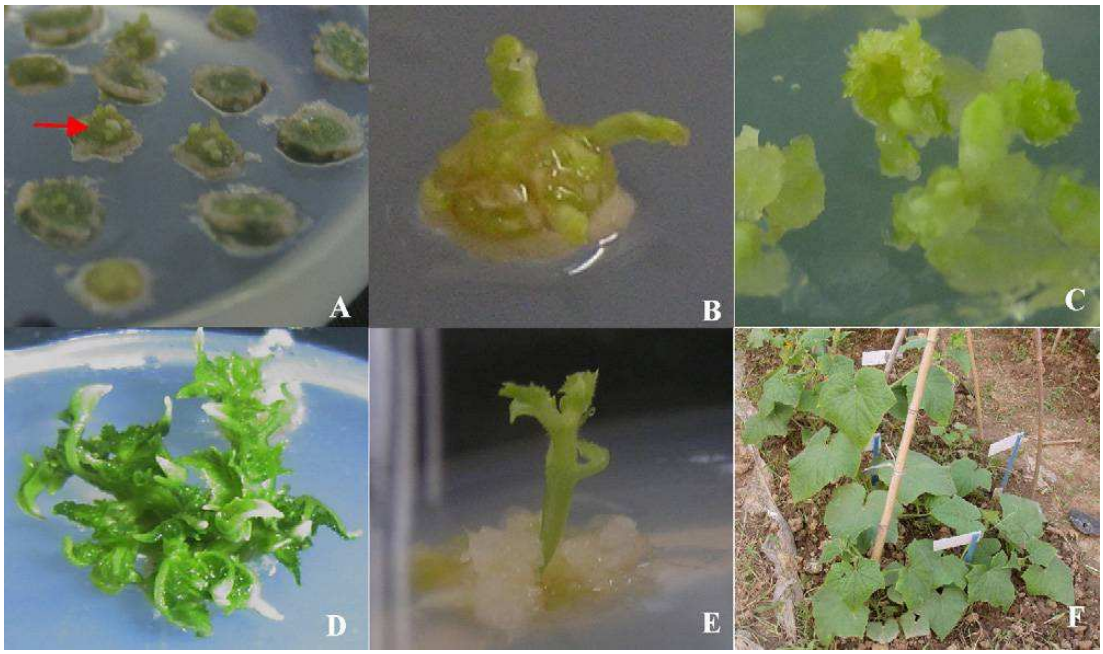
STUDY ON OPTIMIZING DH PRODUCTION OF CUCUMBER BY GYNOGENSIS SYSTEM AND *BRASSICA* BY ANDROGENESIS SYSTEM



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Preface

I received several proposals for different subjects in vegetable breeding from Mrs. Albertien Kijne. I really think the subject “Improve Dihaploidisation protocols for vegetable crops” is intriguing. So I enthusiastically chose this subject as my thesis topic.

The following thesis, while an individual work, benefited from several people.

I would like to thank Mrs. Ellen Rietveld, who is my supervisor at Syngenta. She offered me the research and working opportunity in the vegetable breeding support group. She not only taught me sufficient background knowledge on the cell biology, but also influenced me with her active and passionate attitude on work. I also would like to thank Mrs. Fenna Venema, who is the leader of the group and other group members who all helped me on my experiments.

In particularly, I would like to thank my supervisor of Van Hall Larenstein, Mr. Bernard Gildermasher. He spent time to visit me in the company Syngenta, and gave me a lot of constrictive suggestions on my thesis.

In addition, many thanks for Mrs. Albertien Kijne who gave me the information about this subject and made great efforts on helping me with the tracking difficult problems before I went to the company.

To sum up, I sincerely thank for the great help from all of you. Thank you very much!

***The picture used in the cover page was from Diao *et al*., 2009: Embryogenesis and regeneration of plantlets of cucumber.**

Summary

In the experiment on gynogenesis of cucumber, the different hormones combination and different concentrations of jasmonic acid were investigated on their effect on shoot formation in certain chosen genotypes of cucumber (*cucumis sativus* L) by ovary culture. The results show unexpected shoot formation from all hormone combinations, only few combinations gave shoot but with low efficiency. Moreover, the results show the Miller medium is better to be used as the basic medium compared with MS medium. The results also indicate that jasmonic acid seems to have effects on the shoot formation in the lower concentrations (0.1 µmol and 0.01 µmol).

In the experiment on androgenesis of *Brassica*, the effects of jasmonic acid concentration were tested. By comparison to the results rising from different JA concentrations, 1 µmol JA gave best shoots/anther frequency during the embryogenesis in the genotype of white cabbage, 0.05 µmol JA gave highest score of shoots/anther in the genotype of cauliflower. The shoot formation from embryos was counted after 1st subculture, showing the highest shoot formation frequency from embryo presents in the 0.01µmol JA in white cabbage and 0.05 µmol in cauliflower, respectively. However, due to the limitation of time and data, the results obtained so far are not definite enough to draw clear conclusion on the effects of JA concentrations on the androgenesis of *Brassica*.

709 13

40 0 13

710 13

46 0 13

711 13

54 0 13

713 13

52 0 13

714 13

40 4 0.1 13

715 13

37 0 13

716 13

33 0 13

717 13

42 0 13

Grand total 13

685 13

6 0.9 13

Medium 14

(Miller30) 14

#cucumbers 14

#shoots 14

%S/C 14

0.8BAP/0.2IAA 14

48 14

1 14

2.0 14

0.8BAP/0.4IAA 14

40 14

2 14

5.0 14

1.0ZEA/0.2IAA 14

46 14

0 14

0 14

1.0ZEA/0.2IAA/5Ag 14

46 14

0 14

0 14

1.0ZEA/0.4IAA 14

44 14

1 14

2.2 14

2.0ZEA/0.2IAA.....14

40 14

0 14

0 14

0.02TDZ/0.2IAA.....14

48 14

2 14

4.2 14

MS medium which is rich in salts is being used for many crops (Murashige *et al*., 1962, Appendix 3). The same combinations of hormones were also tested with the MS medium; the results show no shoot was formed.(table 9).....14

Medium 14

(MS30) 14

#cucumbers 14

#shoots 14

%S/C 14

0.8BAP/0.2IAA.....14

48 14

0 14

0 14

0.8BAP/0.4IAA.....14

58 14

0 14

0 14

1.0ZEA/0.2IAA.....14

29 14

0 14

0 14

1.0ZEA/0.2IAA/5Ag.....14

46 14

0 14

0 14

1.0ZEA/0.4IAA.....14

50 14

0 14

0 14

2.0ZEA/0.2IAA.....14

39 14

0 14

0 14

0.02TDZ/0.2IAA.....15

28 15

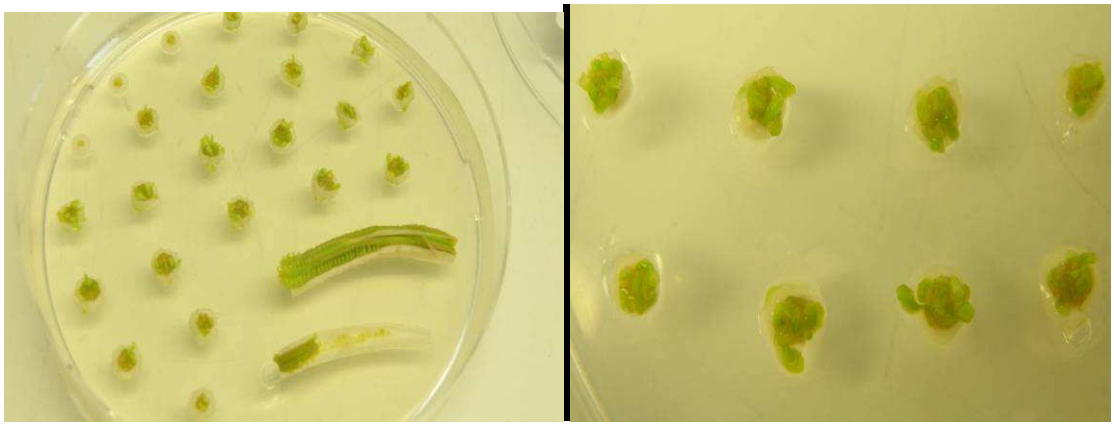
0 15

0 15

At the first stage of ovule development (after one week of culture), the ovary sections or ovary longitudinal slices all showed vigorous responses from both group (Miller and MS). The ovules swelled some stretched out and becoming visible by eyes. 15

TDZ+IAA and BAP+IAA treatment gave yellowish structures by compared with ZEA+IAA which arose greener structure. The ovary sections from the medium with silver-nitrate were greener, with promising swelling of ovules and some differentiation (Picture 6), but no shoot obtained later. Conversely those ovary slices treated by hormones but without silver-nitrate; became yellow-brown with little swelling of ovules and lately shoots did appear in some of hormone combinations with Miller medium. (Table 8) 15

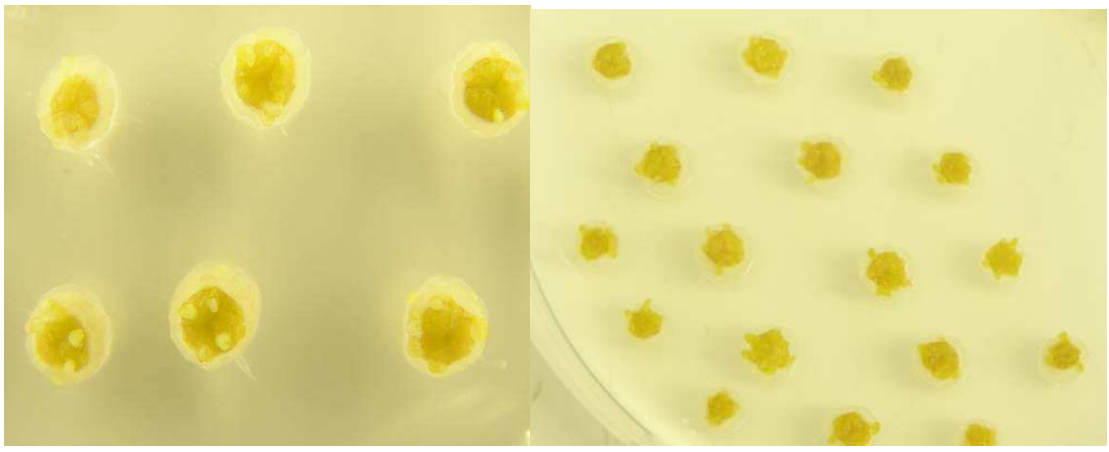
Picture 5: Cucumber ovaries after two weeks 15



15

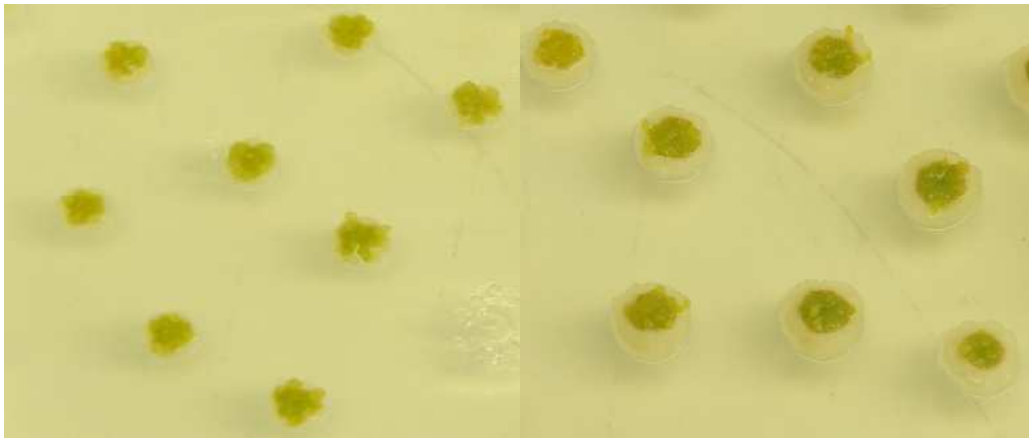
MS30+1.0ZEA/0.2IAA+5Agno3

Miller30+1.0ZEA/0.2IAA+5Agno3.....15



15

Miller30+0.8BAP/0.2IAA **Miller30+0.02TDZ/0.2IAA 15**



concentration	**27**
RPL29	**27**
RPL30	**27**
RPL31	**27**
RPL32	**27**
RPL33	**27**
RPL34	**27**
RPL35	**27**
RPL36	**27**
RPL37	**27**
RPL38	**27**
RPL39	**27**
RPL40	**27**
RPL41	**27**
RPL42	**27**
TOTAL	**27**
0JA (CON)	**27**
1	**27**
2	**27**
0	**27**
1	**27**
3	**27**
0	**27**
0	**27**
0	**27**
4	**27**
20	**27**
20	**27**
0	**27**
0	**27**
2	**27**
53	**27**
1JA	**27**
11	**27**
3	**27**
1	**27**
1	**27**
3	**27**
0	**27**
1	**27**
1	**27**
14	**27**

15	**27**
15	**27**
1	**27**
2	**27**
3	**27**
71	**27**
0.5JA	**27**
2+4	**27**
5	**27**
0	**27**
0	**27**
5	**27**
0	**27**
0	**27**
1	**27**
7	**27**
14	**27**
14	**27**
0	**27**
1	**27**
0	**27**
50	**27**
0.1JA	**27**
2+1	**27**
0	**27**
0	**27**
0	**27**
0	**27**
2	**27**
1	**27**
0	**27**
4	**27**
7	**27**
7	**27**
1	**27**
0	**27**
4	**27**
40	**27**
0.05JA	**27**
6	**27**
1	**27**
1	**27**
0	**27**
11	**27**

11	**27**
2	**27**
2	**27**
5	**27**
0	**27**
0	**27**
0	**27**
0	**27**
0	**27**
39	**27**
0.01JA	**27**
4	**27**
0	**27**
0	**27**
0	**27**
1	**27**
1	**27**
1	**27**
0	**27**
3	**27**
3	**27**
3	**27**
1	**27**
0	**27**
0	**27**
17	**27**
Total of RPL	**27**
26+5	**27**
11	**27**
2	**27**
2	**27**
23	**27**
14	**27**
5	**27**
4	**27**
37	**27**
59	**27**
3	**27**
18	**27**
3	**27**
9	**27**

RPL1	**27**
RPL2	**27**
RPL7	**27**
RPL8	**27**
RPL22	**27**
RPL23	**27**
RPL24	**27**
RPL25	**27**
RPL26	**27**
RPL27	**27**
RPL28	**27**
TOTAL	**27**
0JA (CON)	**27**
7	**27**
1	**27**
5	**27**
0	**27**
3	**27**
3	**27**
3	**27**
0	**27**
0	**27**
1	**27**
4	**27**
21	**27**
1JA	**27**
3	**27**
9	**27**
7	**27**
0	**27**
1	**27**
1	**27**
1	**27**
0	**27**
0	**27**
2	**27**
0	**27**
24	**27**
0.5JA	**27**
6	**27**
3	**27**
7	**27**
0	**27**
1	**27**

1	**27**
1	**27**
2	**27**
1	**27**
2	**27**
4	**27**
28	**27**
0.1JA	**27**
6	**27**
3	**27**
6	**27**
0	**27**
5	**27**
5	**27**
5	**27**
3	**27**
0	**27**
0	**27**
1	**27**
25	**27**
0.05JA	**27**
2	**27**
3	**27**
6	**27**
0	**27**
1	**27**
1	**27**
1	**27**
1	**27**
0	**27**
4	**27**
1	**27**
23	**27**
0.01JA	**27**
0	**27**
11	**27**
11	**27**
0	**27**
3	**27**
3	**27**
3	**27**
1	**27**
1	**27**
5	**27**

0	**27**
35	**27**
Total of RPL	**27**
24	**27**
30	**27**
42	**27**
0	**27**
14	**27**
14	**27**
14	**27**
7	**27**
2	**27**
14	**27**
10	**27**

Specific terms

DH: double haploid Haploids are autonomous, sporophytic plants that have gametophytic chromosome number because they originate from a gametic cell in the embryo sac or in the pollen gain. (Jain *et al.*, 1996).

Zeatin: a plant hormone, a member of the plant growth hormone family knownas cytokinins.

TDZ: short for Tidiazuron a plant hormone, a member of the plant growth hormone family known as cytokinins.

BAP: a plant hormone, a member of the plant growth hormone family known as cytokinins

IAA: a plant hormone, a member of the plant growth hormone family known as auxin.

MS: short for Murashige & Skoog, a common medium used in tissue culture. (Murashige *et al.*, 1962)

Ag: short for $AgNO_3$ is used in plant tissue as an ethylene antagonist. (Beyer et al., 1976)

JA: short for Jasmonic acid, considered to be a new plant growth regulator. Jasmonic acid and his derivates are widely distributed among higher plants. They can influence processes like fruit ripening, production of viable pollen, root growth and plant resistance to insects and pathogens. (Meyer *et al*., 1984)

Chapter 1. Introduction

Syngenta is one of the world's leading companies with more than 25,000 employees. "Bringing plant potential to life" is the purposes of the company.[1] Syngenta has two main business lines, crop protection and seeds production. The innovation on plant breeding is helping to improve the quality of crops and obtaining new varieties. Producing Doubled Haploid is a relatively new technology using for the company especially producing DH[2] vegetable crops. Inducing haploids is used for the development of commercial vegetable hybrids.

DH lines are uniform, where all genes are presented homozygous. The importance of pure lines not only presented in increasing efficiency of selecting certain features but also in marker development. Ovary culture and anther culture have been two of the main approaches which are used in commercially producing homozygous plants and genetic studies.

The company started to produce DH cucumbers 2 years ago, but was not successful due to the low efficiency of shoots obtained. Cucumber (*Cucumis sativus* var. *satvus* L) is a member of family *Cucurbitacea*. Working with pure lines is important for the breeding in order to gain strength in the North European market, and marker assisted breeding. It takes 6 to 8 years to get parental lines needed for hybrid breeding in the way of traditional inbreeding. However, haploid induction through *in vitro* gynogenesis in cucumbers makes it much more efficient on getting pure lines. The ovary culture of cucumber is used in the DH production of cucumbers with using unfertilized ovaries. The successful development of haploid cucumber plants via *in vitro* gynogenesis has been described by Gemes *et al*. (2002) and by the patent of Nunhem Zaden B.V. (1996). Although the ovary culture has been used to produce haploids and double haploids in cucumber (Gemes et al., 2002), the regeneration of DH lines is still difficult. (Diao *et al*., 2009), of the 33dihaploid plants, 17(51.5%) were identified as double haploids.

This research on DH production of cucumber follows up of the experiments started two years ago which was done last year by Ellen Rietveld. The results from last two years showed that the different ovary responses depended on temperature, genotype, media variables, and way of cutting.
This research mainly focuses on testing the effect of different hormone combinations and different concentrations of jasmoinc acid.

[1] http://www2.syngenta.com/en/about_syngenta/

[2] Double haploids

The previous experiments showed that the balance of the cytokinins[3] and auxins[4] was essential for getting haploid cucumber shoots (Nuhnem 2007). Different concentration of Tidiazuron (TDZ) was used by Diao *et al.* (2009) and Geme *et al.* (2002) in the induction medium. Silver nitrite[5] has been tested by Diao *et al.* (2009) in the concentrations of 10mg/l with MS as basic medium, and the result showed higher embryo formation frequency compared to the controls. Therefore 5mg/l $AgNO_3$ was added in one of the combinations. The reason for using jasmonic acid is because it has been found that in low concentrations (0.01 – 1 µMol) have a hormonal function and stimulates cell division (Toro *et al*., 2002).

Unlike cucumber, the DH production protocol of *Brassica* (*Brassica oleracea*) has already been established. However, the productions of DH on some Brassica genotypes are not efficient. Here, jasmonic acid may be an improvement for recalcitrant genotypes. Besides the reason of increasing the efficient of selecting complex traits and the importance of marker development, DH production of *Brassica* also shorten the time of getting pure homozygous lines from 10 or more than 10 years to 2 years. Furthermore, DH line is important for developing multi-genetic traits, such as one genotype combined with more than one or two resistance genes on diseases.

The research was done in the company Syngenta, from February to June 2010. The research was done to optimize the DH production of cucumber and *Brassica*. There are two different experiments for the research; described by “A” and “B” for the cucumber and *Brassica*. The experiment of hormones combinations on cucumber is described as “A1”, experiment of different concentrations of Jasmonic acid described as “A2”. The experiment on effect of different concentrations of jasmonic acid on Brassica is described as “B”. In this report, the research objective and research questions are defined in the second chapter. In the next chapter, the detailed experimental information is described. In the third chapter, information of materials chosen for the experiments are illustrated and the protocol or processes of experiments are described as well. The results of all experiments can be found in the chapter four; the analysis based on the results is elaborated. In last chapter the results discussed, the research questions are answered and conclusions are drawn. Furthermore, the recommendations about these experiments can also be found in this part. The literature details are in the part of references. The supplementary data which are relevant to each experiment can be found in the part of appendix.

[3] One type of plant hormones, cytokinins are derived from adenine and produce two immediate effect on undifferentiated cells: the stimulation of DNA synthesi and increased cell division (Kumar et al., 2009 Plant tissue culture).

[4] One type of plant hormones, auxins promote adventitious root development (Kumar et al., 2009 Plant tissue culture).

[5] AgNo3 used in plant tissue culture as an ethylene antagonist (Beyer, 1976).

Chapter 2. Research objectives and experimental details

The research objectives are clarified and research questions are defined in the flowing paragraphs.

2.1 Experiment A

Experiment A contains experiments on cucumbers.

2.1.1 Research Objectives

To develop an effective and official system of producing DH cucumber by gynogenesis system.

2.1.2 Research Question

Main question:
What kind of factors can improve the DH production of gynogenesis system of cucumber?

Sub questions:
1. Which kind of hormones or variable conditions can improve the protocol of DH production of gynogenesis system of cucumber?
2. Do different concentrations of jasmonic acid have influence on gynogenesis of cucumber?

2.2 Experiment B

Experiment B is experiment on *Brassica*.

2.2.1 Research Objectives

To improve DH production of androgenesis system of *Brassica*.

2.2.2 Research Question

Main question:
Does jasmonic acid improve the production of DH of *Brassica* by androgenesis?

2.3 Experimental details

There are two different experiments for the research; described by "A" and "B"

A. Experiment (on cucumber)
   A1: Experiment with all different hormones which are added in the standard Miller and MS medium, zeatin+IAA, TDZ[6]+IAA, BAP+IAA. There is one combination with $AgNO_3$.
   A2: The experiment aims to test the effect of different concentrations of jasmonic acid (three different concentrations and one control) which is added in the standard Miller medium with 0.8 BAP.

B. Experiment (on *Brassica*): the experiments is designed to test whether jasmonic acid improves the induction of embryo's formation and further development of embryo's into plants of white cabbage and cauliflower in one genotype 10002of white cabbage and genotype 10015 of cauliflower.

[6] Short for Tidiazuron

Chapter 3. Materials and Methods

All medium and stock solutions were prepared by the BSF [7] medium kitchen department and preparation of petridishes to provide for medium making was done by our group BSV[8].

3.1 Experiment A (Cucumbers)

3.1.1 Plant materials

16 genotypes were used in these experiments. Plants are delivered from De Lier, where plants are grown in hydro-culture in greenhouse under certain controlled conditions, light condition; 12/12h (light/dark), SONT-T(600watt), temperature with 23℃/21℃(light/dark). Un-pollinated ovaries (small cucumbers) are harvested from the plants 1 to 3 days before the flower opens. The 16 genotypes were chosen by their genetic background; all these genotypes are based upon crosses with varieties of competitive companies', and expected to have gynogenetic competence, because the competitive companies have been used this method for long time to produce the cucumber parental lines, and thus hybrids DH lines are original from the gynogenesis process seem to have a high competence for the gynogenesis, and thus give high responses. In this experiment all genotypes were used cucumbers from A and B compartment (A with using chemical spraying against plant diseases, B without).
The table below is the information about genotypes used in different experiments.

[7] BSF is short for Breeding Support Flower department.

[8] BSV is short for Breeding Support Vegetable department.

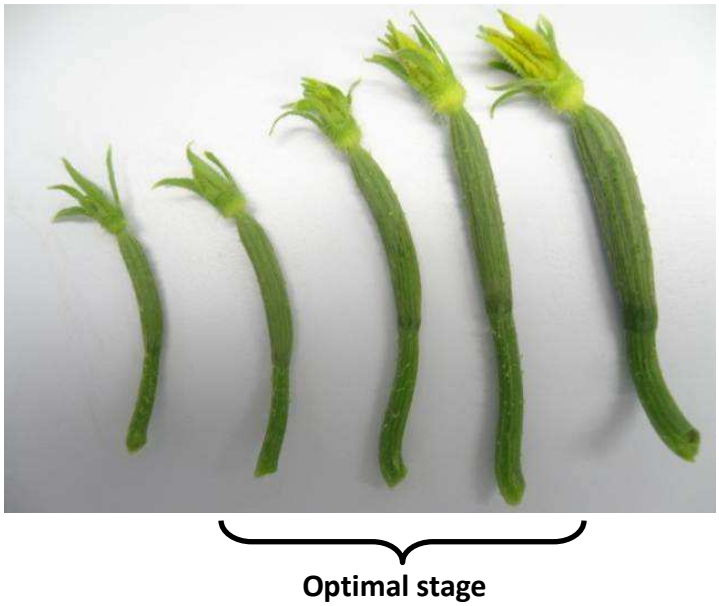
Table 1: The list of genotypes used in the experiments

Code 2010	Used in the experiments
700	A1 A2
701	A1
702	A1
703	A1 A2
704	A1
705	A1
707	A1
708	A1
709	A1
710	A1 A2
711	A1
713	A1
714	A1
715	A1
716	A1
717	A1 A2

Un-pollinated ovaries (small cucumbers) were harvested from plants 1 to 3 days before the flower opened. The picture below showed the different stages of small cucumbers, and the three cucumbers in the middle are in the optimal stage[9].

[9] The optimal stage has been revealed after observation by Gemes et al., (2009) that the ovules are at the best stage for haploid induction just before anthesis (6h), at the time of embryo sac usually contains all eight nuclei, and cellulaization is in prograss.

Picture 1: The different stages of small cucumbers (un-pollinated ovaries, made by Ellen Rietveld, 2009)



3.1.1.1 Experiment A1 (Experiment on different hormones)

In this experiment all genotypes were used cucumbers from A and B compartment (A with using chemical spraying against plant diseases, B without) were combined together. Two basic medium were used; Miller medium and MS medium. Different hormone combinations were added in both basic medium as table 2 showed below.

Table2: The different hormone combinations

0.8BAP/0.2IAA	**0.8BAP/0.4IAA**	**1ZEA/0.2IAA**	**1ZEA/0.2IAA/5Ag**	**1ZEA/0.4IAA**	**2ZEA/0.2IAA**	**0.02TDZ/0.2IAA**

3.1.1.2 Experiment A2 (Experiment on different concentration of JA)

Four genotypes were used in this experiment with two treatments A and B (A with using chemical spraying against plant diseases, B without).
The medium of this experiment used the basic medium Miller medium with 4 different concentrations of jasmonica acid (one is control)

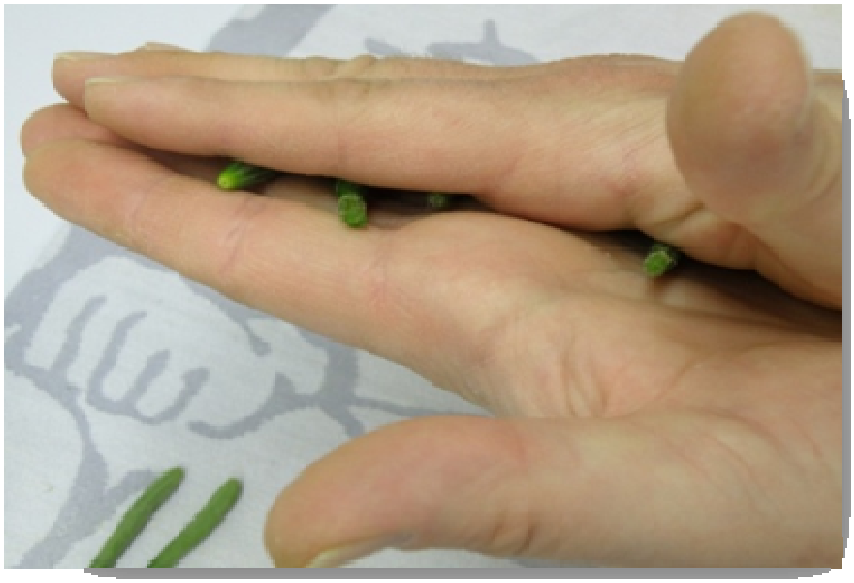
Table 3: the different concentration of jasmonic acid (JA)

1JA (1µmol/liter)	**0.1JA (0.1µmol/liter)**	**0.01JA (0.01µmol/liter)**	**Without JA (Control)**

3.1.2 Treatment before sterilization

Small cucumbers were rolled by hands on a cotton cloth or between hands, in order to unfasten the shell from the inner tissue. Depending on the size of the cucumber, rolling was done about 1 minute.

Picture2: Rolling cucumbers before disinfection (made by Ellen Rietveld, 2009)



3.1.3 Sterilization of ovaries (small cucumbers)

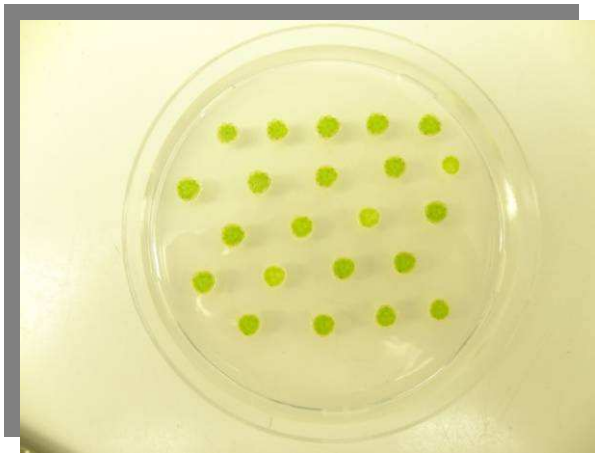
After rolling, small cucumbers were disinfected 1 minute with 70% ethanol, followed by 25 minutes 1.5 % chloride solution (37.5ml Glorix per 100ml bidest). Then those pre-treated cucumbers were rinsed three times 10 minutes with sterile water. During disinfection, pots were placed on a rotary shaker (100rpm).

3.1.4 Ovary culture

Ovaries were cut into sections with one or more unfertilized ovules or longitudinal slices, and then the sections were placed on the culture medium in petridish.

Picture3: Cutting of small cucumbers (Un-pollinated ovaries)

Picture4: The cucumber discs sections.



Picture 5: The cucumber longitudinal slices

(The picture3, 4 and5 were made by Ellen Rietveld, 2009)

3.1.5 Ovary sections development

After ovary culture, plates were incubated at 35℃ for 63h -90h (2-4 days range has no differences on the responses of ovary sections), followed by 12-11 at 21℃ with 16h day and 8h night increased the induction of embryos formation. Then the plates were placed in the grow chamber at 25℃-28℃ with 16h day and 8h night for growth of the culture.

The shoots or callus-like structures obtained from the ovary sections were transferred in to the RU medium (Appendix 1), which has effects on the shoots development.

3.2 Experiment B (*Brassica*)

3.2.1 Plant materials

For this experiment two different species were used, both with one genotype, white cabbage (code of genotype is 10002) and cauliflower (code of genotype is 10015).

Donor plants were grown under controlled conditions at 15 ℃ /13 ℃ , ± 10,000lux(SON-T and HPI-T illumination), with 16 hours light and 8 hours of darkness. Those plants were watered three times a week and fertilized with a solution of 15:5:25, NPK.

In the experiment, six different concentrations of JA were used (as the table 4 shows below) and the layout of the concentrations in the replica can be seen from table 5.

Table4: the different concentration of JA

1JA(1µmol/liter)	**0.1JA (0.1µmol/liter)**	**0.1JA (0.1µmol/liter)**	**0.1JA (0.1µmol/liter)**	**0.1JA (0.1µmol/liter)**	**Without JA(Control)**

Table5: The layout of the different concentrations in the replica

con	**1JA**	**0.5JA**	**0.1JA**	**0.05JA**
con	**1JA**	**0.5JA**	**0.1JA**	**0.05JA**
con	**1JA**	**0.5JA**	**0.1JA**	**0.05JA**
con	**1JA**	**0.5JA**	**0.1JA**	**0.05JA**
0.01JA	**0.01JA**	**0.01JA**	**0.01JA**	

3.2.2 Sterilization of buds

Small flowers (unopened flower buds) of *Brassica* were collected from plants. Buds were disinfected 1 minute with 70% ethanol, followed by ±15-20 minutes 1% chloride solution(25ml Glorix per 100ml bidest). Then they were rinsed three times with sterile water. Sterilized buds were stored in a closed container at low temperature(±10℃) until they would be used for anther culture.

3.2.3 Anther culture

The anther cultures were done under micro-scope (0.8-10×). Buds were selected with specific ratio of petal/anther length of 0.8 to 1.3 and used for plating. Anthers were plated in the replica dishes (Greiner) with induction medium containing jasmonica acid). The six anthers from one bud were divided over 6 wells[10].

Table6: The outline of the replica dishes

B1-B6	**B1-B6**	**B1-B6**	**B1-B6**	**B1-B6**
B7-B12	**B7-B12**	**B7-B12**	**B7-B12**	**B7-B12**
B13-B18	**B13-B18**	**B13-B18**	**B13-B18**	**B13-B18**
B19-B24	**B19-B24**	**B19-B24**	**B19-B24**	**B19-B24**
B1-B6	**B7-B12**	**B13-B18**	**B19-B24**	

Outline of the replica; B1-B6 indicates the anthers of bud no.1 to bud no.6. B7-B12 indicates the anthers of bud no.7 to 12, etc.

In total 6 anthers were plated per well (144 anthers per plate), 7 plates of white cabbage and 7 plates of cauliflower. Anthers were plated with their flat outside on the medium.

3.2.4 Embryo development

The replicas were sealed with nescofilm and incubated at 35□ for 24h, after that, the plates were placed in the dark at 23□.

Four weeks after initiation, embryos with sizes of ±2mm were transferred to RU medium and incubated at 23□, 16 h photoperiod, ±2000 lux.

[10] Wells here means each small space on the replica dishes

Chapter 4. Results and Discussions

4.1 Experiment A (Cucumber)

The experiment A2 started at week 6 2010 till week 9 2010. And the experiment A1 was done from week 9 2010 to week 10 2010. After 3-4 weeks, the shoots formatted from ovaries can be seen.

4.1.1 Experiment A1

In total 685 un-pollinated ovaries (small cucumbers) were used for the test of the different hormone combinations, described as experiment A1. Fifteen genotypes were used; six shoots were harvested from three different genotypes. The results are shown in the Table 7 below.

Table 7: Overview of genotype and number of shoots obtained in experiment A1

Genotypes	#cucumbers	#shoots	%S/C
700	42	1	2.3
701	48		0
702	28		0
703	51		0
704	44		0
705	34		0
707	57		0
708	37	1	2.7
709	40		0
710	46		0
711	54		0
713	52		0
714	40	4	0.1
715	37		0
716	33		0
717	42		0
Grand total	685	6	0.9

The Miller medium, which is a very common medium with low salts concentration

described in the patent of company Nunhems (the recipe of Miller medium can be seen in Appendix 2), was used as the basic medium. Seven different combinations of cytokines and Auxin were tested and for (1.0ZEA+0/2IAA) 5mg/l $AgNO_3$ was added to enhance embryonic formation (Diao *et al.*, 2009). The combination of 0, 8BAP/0,4IAA showed a relatively high shoot formation frequency at 5.0%.(table 8)

Table 8: Overview of the tested different hormones combination with using the standard Miller medium

Medium (Miller30)	#cucumbers	#shoots	%S/C
0.8BAP/0.2IAA	**48**	**1**	**2.0**
0.8BAP/0.4IAA	**40**	**2**	**5.0**
1.0ZEA/0.2IAA	**46**	**0**	**0**
1.0ZEA/0.2IAA/5Ag	**46**	**0**	**0**
1.0ZEA/0.4IAA	**44**	**1**	**2.2**
2.0ZEA/0.2IAA	**40**	**0**	**0**
0.02TDZ/0.2IAA	**48**	**2**	**4.2**

MS medium which is rich in salts is being used for many crops (Murashige *et al.*, 1962, Appendix 3). The same combinations of hormones were also tested with the MS medium; the results show no shoot was formed.(table 9)

Table 9: Overview of the tested different hormones combination with using the standard medium MS medium

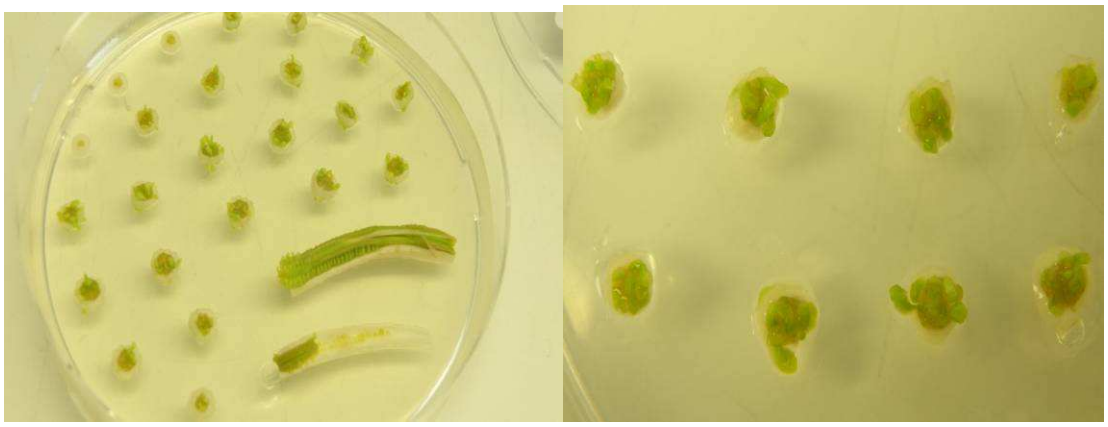
Medium (MS30)	#cucumbers	#shoots	%S/C
0.8BAP/0.2IAA	**48**	**0**	**0**
0.8BAP/0.4IAA	**58**	**0**	**0**
1.0ZEA/0.2IAA	**29**	**0**	**0**
1.0ZEA/0.2IAA/5Ag	**46**	**0**	**0**
1.0ZEA/0.4IAA	**50**	**0**	**0**
2.0ZEA/0.2IAA	**39**	**0**	**0**

0.02TDZ/0.2IAA	**28**	**0**	**0**

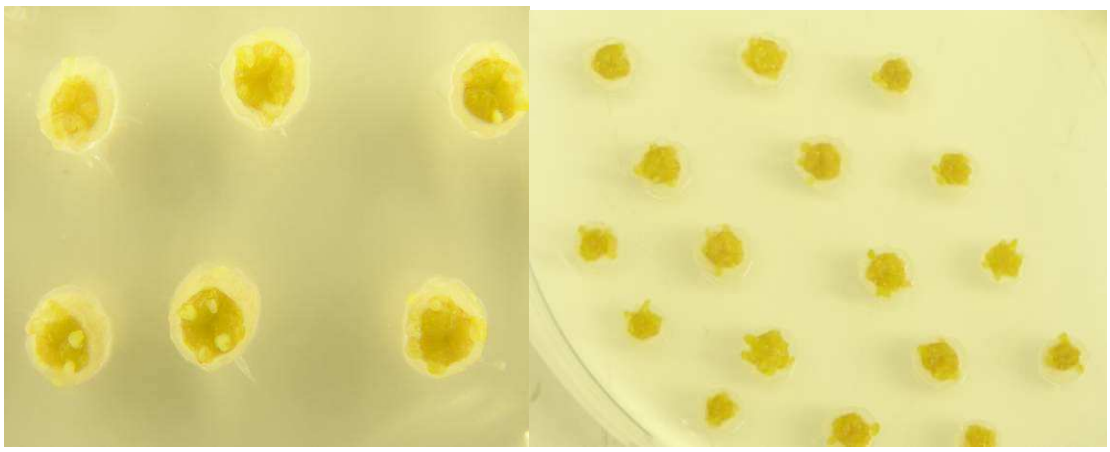
At the first stage of ovule development (after one week of culture), the ovary sections or ovary longitudinal slices all showed vigorous responses from both group (Miller and MS). The ovules swelled some stretched out and becoming visible by eyes.

TDZ[11]+IAA and BAP+IAA treatment gave yellowish structures by compared with ZEA+IAA which arose greener structure. The ovary sections from the medium with silver-nitrate were greener, with promising swelling of ovules and some differentiation (Picture 6), but no shoot obtained later. Conversely those ovary slices treated by hormones but without silver-nitrate; became yellow-brown with little swelling of ovules and lately shoots did appear in some of hormone combinations with Miller medium. (Table 8)

Picture 5: Cucumber ovaries after two weeks

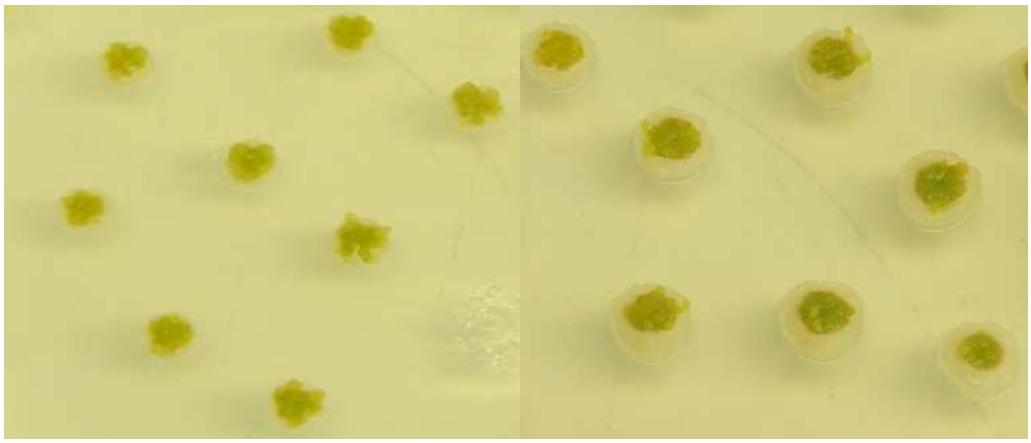


MS30+1.0ZEA/0.2IAA+5Agno3 **Miller30+1.0ZEA/0.2IAA+5Agno3**



Miller30+0.8BAP/0.2IAA **Miller30+0.02TDZ/0.2IAA**

[11] Short for Tidiazuron



Miller30+1.0ZEA/0.2IAA **Miller30+2.0ZEA/0.2IAA**

(Picture 5 was made by Xiaoqian Shi in April 2010)

The experiment described by Diao *et al.*, (2009), which used 10mg/l silver nitrate with MS as the basic medium; $AgNO_3$ shortens the time of embryo induction and increases embryo yield. Conversely, in the experiment A1, no embryo was formed in $AgNO_3$ containing the medium. This could be explained by a not optimal $AgNO_3$ concentration (only one concentration of 5mg/l was tested) and probably not optimal or incomplete hormone combination.

Miller medium together with MS medium in different hormones combination were tested as the tables (8, 9) showed above, both all give promising responses (swelling ovules), however there is no shoot was initiated on the MS medium, indicating MS medium is not appropriate to be the initiation medium with adding the hormones combinations.

Although some shoots were obtained from the three (0.8BAP/0.2IAA, 1.0ZEA/0.4IAA, 0.02TDZ/0.2IAA) of the hormone combinations with Miller medium, the efficiencies were too low to draw a definitive conclusion. Furthermore, those shoots developed in an abnormal way, sometimes with a callus phase, and more slowly than described in literature, where 4-5 weeks shoots are obtained after initiation is mentioned (Diao *et al.*, 2009). No “true” embryonic development was obtained. Therefore it remains to be questionable whether we deal with true “double haploids” or just somatic regenerants.

4.1.2 Experiment A2

In this experiment of testing effects of different concentration of JA, JA was added to the standard initiation medium (Miller 30 with 0.8 g/l BAP)

Four genotypes were chosen for this experiment; 3 out of 4 produced shoots except the genotype 717 gave no shoot (Table 10), thus this genotype seems to be less responsive.

Table 10: Overview of genotype and shoots obtained

Genotypes	#cucumbers	#shoots	%S/C
700	**154**	**15**	**9.7**
703	**180**	**4**	**2.2**
710	**160**	**13**	**8.1**
717	**148**	**0**	**0**
Grand total	**642**	**32**	**5.0**

The effect of different concentrations of JA on shoots formation frequency is presented in the table 11 below. The results show all JA concentrations including control medium induced shoots formation. Concentration of 0.01 JA shows the highest frequency of shoot formation. In the light of these results, JA seems to stimulate the shoot formation by comparison with the shoots formation frequency on the control medium. Particularly, the lower concentration of JA induces the higher efficiency of shoot formation.

Table11: Overview of different concentration of JA and shoots obtained

concentration	#cucumbers	#shoots	%S/C
0JA(con)	**168**	**6**	**4.1**
1JA	**163**	**7**	**4.3**
0.1JA	**159**	**8**	**5.0**
0.01JA	**158**	**11**	**6.9**

However, based on these results it is still conclusive to prove that JA stimulates shoot formation. Also, the obtained data in this experiment are not adequate enough to indicate that the lower concentrations of JA are inducing more shoots. Further decreasing JA level and testing more concentrations could confirm its role on the shoot formation.

It was expected the treatment of the donorplants with chemicals (cucumbers from compartment A with chemical treatment) against pest and diseases would have a negative effect on the response of the ovaries, compared to no chemical treatment

(cucumbers from compartment B). However, no significant difference was found; for both conditions ovaries were observed producing shoots (Rietveld, 2009). Consequently, the cucumbers with two treatments (A and B) were mixed and counted together. However, it is not conclusive enough to exclude the possibility that these two treatments have impact on the shoot formation in this experiment. Furthermore, it is also uncertain if JA stimulates the spontaneously regenerates shoots instead of effects on the gynogenesis regeneration. The purity of haploid lines will be known after second regeneration of the obtained shoots.

4.2 Experiment B (*Brassica*)

The experiments were done with two species of *Brassica* (*Brassica oleracea*) , white cabbage and cauliflower (Table 12).

Table 12: The genotype background

Common name	**Cultivar group**	**Genotype used**
White cabbage	**Capitata**	**10002**
Cauliflower	**Botrytis**	**10015**

The number of embryos harvested from each replica plated of white cabbage and cauliflower can be seen in the Appendix 4

The embryos were transplanted in to the Ru30 with 8mg/l glutamine medium in plastic Ecoline cups, after 4weeks the 1st subculture was done and developing structures were transplanted to Ru30 in glass baby jar. The numbers of shoots were counted and most of the structures developed from embryos were embryo like structures (ELS) with shoot initiations which can be regarded as potential “shoots”.

Fourteen plates for genotype of white cabbages were completed. In total 2016 anthers were plated and 216 embryos were harvested.

Table 13: overview of different concentrations of JA tested on the **white cabbage** and number of shoots obtained from the 1st subculture

Concen.	# Anthers	# embryos	%E/A	#shoots	#ELS *	%S/E	%S/A
0JA(CON)	324	53	16.4	6	26	11.3	1.86
1JA	324	71	21.9	5	36	7.0	1.5
0.5JA	324	50	15.4	5	26	10.0	1.5
0.1JA	324	40	12.3	0	26	0	0
0.05JA	324	39	12.0	2	32	5.1	0.6
0.01JA	324	17	5.1	5	16	29.4	1.5

* Embryo-like structure with shoot potential

According to the results, the different efficiencies were calculated (%Embryo/Anther, %Shoot/Embryo and %Shoot/Anther). The control (0 JA) presents the highest frequency 1.86% in ratio of Shoot/Anthers. The medium with 0.01 JA showed the highest efficiency in the terms of S/E ratio 29.4 % among the used concentrations. On the other hand, the highest E/A ratio (21.9%) is observed at the concentration of 1 JA and this ratio decreases with lowing JA concentration. It might indicate that JA stimulates cell division at the early embryonic stage and inhibits cell differentiation during the post embryonic phase. So, it could be a good try to firstly apply high JA at early time point and subsequently switch to low JA in order to maximize S/A %. However, more different JA concentrations need to be tested to get clear tendency on both E/A and S/E.

Eleven plates for genotype of cauliflower plates for genotype of cauliflower were completed. 1584 anthers were plated. 171 embryos were harvested.

Table 14: overview of different concentrations of JA tested on the **cauliflower** and number of shoots obtained from the 1st subculture

Concen.	#Anthers	#embryos	%E/A	#shoots	#EMS *	%S/E	%S/A
0JA(CON)	264	21	8.0	8	18	38.1	3.0
1JA	264	24	9.1	6	19	25	2.3
0.5JA	264	28	10.6	4	12	14.3	1.5
0.1JA	264	25	9.5	9	16	36	3.4
0.05JA	264	23	8.7	10	26	43.5	3.8
0.01JA	264	35	13.3	9	19	25.7	3.4

* Embryo-like structure

In the table above the relatively high frequency of embryos developed from anthers was promoted by the medium with the 0.01 JA. The concentration of 0.05 JA medium showed the high efficiency of shoots formation from embryos. Although the concentration of 0.05 also presented the highest efficiency of shoots formation from same amount of anthers (S/A), the gaps of these efficiencies are not big enough to conclude the differences.

By comparison to the data from white cabbage (Table 13), the relatively high efficiency of shoot formation consistently presents in the low concentration of JA medium. This result coincides with the finding the low concentration of JA enhances root development of *Brassica* and could also significantly increase the shoot formation (Toro et al., 2003). Furthermore it was also described by Ravnikar (1991) low concentration of JA (0.01 and 0.1um) results in the increasing frequency of cell division and microcallus formation. However, because of the limitation of the time and data the experiments on the *Brassica* (white cabbage and cauliflower), it is not enough to show clear differences on the embryo formation and shoots formation among different concentrations of JA, even the obtained data cannot prove whether

low JA concentration has positive effect on the shoot formation in the DH production of *Brassica*. Normally the shoots are counted after 2nd subculture under the full development of embryos or embryo-like structures, but in these experiments, the 1st subculture was applied and there are still many embryo-like structures can or cannot develop into shoots later. This could result in the deviated results, so the large numbers of data are needed to unmask the JA effect on shoot formation.

Chapter 5. Conclusions and Recommendations

5.1 Experiment A (Cucumber)

Experiments A contains two experiments (A1 and A2) about cucumber ovary cultures, both of the experiments showed relatively low shoot formation efficiency. The productivities of shoots obtained from both experiments are not sufficient to develop into a protocol of producing DH cucumber.

5.1.1 ExperimenA1 (hormone combination test)

This experiment is testing effect of different hormone combinations. Based on the results there was no hormones combinations can improve the shoots formation even the DH production. Although some of combination showed shoots obtained, the efficiency is still too low. Compared with Miller medium, MS is not a suitable induction medium used for basic medium. It could be that other hormone combination need to be tested, but it could also be that the applied stress treatment (35C) is not optimal.

5.1.2 ExperimentA2 (jasmonic acid)

The experiment of A2 is testing the effect of jasmonica acid on the DH production on cucumber. The data collected in this experiment show a relatively high shoot formation present at the concentration of 0.01JA; however, it is insufficient to conclude if the JA can improve the shoots formation or even DH production of gynogenesis system of cucumber.

5.2 Recommendations of experiment A

According to the results obtained from experiment A (A1 and A2), there are several improvements were concluded for the future experiments:

1. The Miller medium can be used for the basic medium in the following experiments.
2. More concentrations of different hormones combinations can be tested, especially the combinations which triggered shoots obtained.

3. For further experiments it is necessary to search for other/or extra stress-treatments, in order to trigger the right response of the ovules, like use of 2, 4-D[12] stress, cold treatment or starvation on mannitol medium (no sucrose).
4. For further research JA can be tested on a larger scale and not only in the induction medium but also upon early transfer of cucumber explants 1-2 weeks after initiation of the cultures.

5.3 Experiment B (*Brassica*)

The experiment B is testing the effect of jasmonic acid on producing DH *Brassica*. Although the results from the two experiments of *Brassica* show some different effects among the different concentration of JA, it is still insufficient to conclude whether the JA has effects on the embryos formation or shoots formation because of the limitation of time and data of the experiments. Therefore, the results are limited to indicate if JA can really improve the DH production of DH of *Brassica* by androgenesis.

5.4 Recommendations of experiment B

Jasmonic acid may have effect on embryo formation of shoots formation; however more tests have to be done later. The further research using wider JA concentration range and more genotypes is needed to reveal JA's role on DH production.

[12] a plant hormone, a member of the plant growth hormone family known as auxin. 2, 4-D was found to have effect of stimulating gynogenic development in the high concentration (Alisher et al., 2007)

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Appendix

Appendix I: The recipe of Ru30 medium

	Ru (mg/l)
KNO3	1100
KCl	500
CaCl2	332.16
Ca(NO3)2.4H2O	416.92
MgSO4. 7H2O	732.6
KH2PO4	340
NH4NO3	412
KI	0.83
MnSO4.H2O	16.9
ZnSO4.7H2O	14.3
H3BO3	12.4
Na2MoO4.2H2O	0.25
CuSO4.5H2O	0.25
CoCl2.6H2O	0.025
FeNaEDTA	36.7
L-glutamine*	8
Glycin	2
Folic acid	0.5
Nicotinic acid	5
Thiamine HCl	0.5
Pyridoxine HCl	0.5
myo--Inositol	100
biotine	0.05
sucrose	30g/l
pH	5.7

Appendix II: The recipe of Miller medium

	Miller
KNO3	80
KCl	65
CaCl2	
Ca(NO3)2.4H2O	100
MgSO4. 7H2O	35
KH2PO4	12
NH4NO3	400
KI	0.8
MnSO4.H2O	4.4
ZnSO4.7H2O	1.5
H3BO3	1.6
Na2MoO4.2H2O	
CuSO4.5H2O	
CoCl2.6H2O	
FeNaEDTA	36.7
L-glutamine	0.2
Leucine	0.012
Spermidine	0.01
Glycin	20
Folic acid	
Nicotinic acid	5
Thiamine HCl	9
Pyridoxine HCl	5
***myo*--Inositol**	100
biotine	
sucrose	30
BAP	0,8mg/l
micro agar	6.75

Appendix III: The recipe of MS medium

	MS
KNO3	1900.00
KCl	
CaCl2	332.02
Ca(NO3)2.4H2O	-
MgSO4. 7H2O	180.54
KH2PO4	170.00
NH4NO3	1650.00
KI	0.83
MnSO4.H2O	16.90
ZnSO4.7H2O	8.60
H3BO3	6.20
Na2MoO4.2H2O	0.25
CuSO4.5H2O	0.03
CoCl2.6H2O	0.03
FeNaEDTA	36.70
L-glutamine	0.20
Leucine	0.01
Spermidine	0.01
Glycin	2.00
Folic acid	-
Nicotinic acid	0.50
Thiamine HCl	0.10
Pyridoxine HCl	0.50
***myo*--Inositol**	100.00
biotine	
sucrose	30
BAP	0,8mg/l
micro agar	6.75

Appendix IV: Result of embryo harvested from *Brassica*

Table: The number of embryos harvested from each replica plates (white cabbage)

concentration	RPL 29	RPL 30	RPL 31	RPL 32	RPL 33	RPL 34	RPL 35	RPL 36	RPL 37	RPL 38	RPL 39	RPL 40	RPL 41	RPL 42	TOTAL
0JA (CON)	1	2	0	1	3	0	0	0	4	20	20	0	0	2	53
1JA	11	3	1	1	3	0	1	1	14	15	15	1	2	3	71
0.5JA	2+4	5	0	0	5	0	0	1	7	14	14	0	1	0	50
0.1JA	2+1	0	0	0	0	2	1	0	4	7	7	1	0	4	40
0.05JA	6	1	1	0	11	11	2	2	5	0	0	0	0	0	39
0.01JA	4	0	0	0	1	1	1	0	3	3	3	1	0	0	17
Total of RPL	26+5	11	2	2	23	14	5	4	37	59	3	18	3	9	

*RED number is the embryo which got contaminated

Table: The number of embryos harvested from each replica plates (cauliflower)

concentration	RPL1	RPL2	RPL7	RPL8	RPL22	RPL23	RPL24	RPL25	RPL26	RPL27	RPL28	TOTAL
0JA (CON)	7	1	5	0	3	3	3	0	0	1	4	21
1JA	3	9	7	0	1	1	1	0	0	2	0	24
0.5JA	6	3	7	0	1	1	1	2	1	2	4	28
0.1JA	6	3	6	0	5	5	5	3	0	0	1	25
0.05JA	2	3	6	0	1	1	1	1	0	4	1	23
0.01JA	0	11	11	0	3	3	3	1	1	5	0	35
Total of RPL	24	30	42	0	14	14	14	7	2	14	10	