NATO SCIENCE FOR PEACE AND SECURITY PROGRAMME

REINTEGRATION GRANT

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SECOND INTERIM REPORT

Return Fellow and Host Institution Director

The Return Fellow and Host Institution Director should submit this report jointly not later than the end of the second year of the Reintegration Grant

1. Project Title

Implementation of Novel Biotechnological Methods Towards Food Security

2. Participants

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3. Scientific Report

A joint statement by the Return Fellow and Host Institution Director on the scientific activity undertaken and the results achieved with the support provided by the NATO Reintegration Grant.

During the period July 2007 - June 2008, different scientific activities were undertaken in the Faculty of Agriculture, Goce Delcev University - Stip[1], regarding the project "Implementation of Novel Biotechnological Methods Towards Food Security" awarded by NATO Public Diplomacy Division, Collaborative Programmes Section under reference EAP.RIG.982433 and accepted under the conditions stated in the letter of award by the Return Fellow and Director of Host Institution on 13 July 2006.

In the second experimental year different project activities were undertaken as continuation of the project activities in 2006/07. The project activities can be divided into two directions, the first one is characterisation of androgenic pepper lines obtained during the first experimental year and the second one is continuation of induction of androgenesis is different pepper genotypes.

[1] ***Goce Delcev University - Stip was founded in March 2007 from the Government of Republic of Macedonia. In September 2007, the Institute of Southern Crops - Strumica joined the University and was transformed into Faculty of Agriculture in Strumica within the University. All the activities and responsibilities that were undertaken in the Institute of Southern Crops - Strumica are continuing to be implemented within the Faculty of Agriculture. Prof. Dr. Saša Mitrev, who was director of the Institute of Southern Crops - Strumica as host institution for this project and responsible for its implementation, currently is the Rector of Goce Delcev University and responsible for all activities of the University.***

The fully regenerated plants that were obtained via the process of androgenesis in the first experimental year were grown under greenhouse conditions for observation and seeds collection. The very important observation was that not all regenerated plants were fertile and produce fruits with seeds (Table 1). The seeds from fertile fruits were collected separately, but taking in consideration, that the androgenic plants were agril cover protection, and from practical breeding reasons, seeds from the same androgenic plants were joined together and the androgenic lines were created (Table 2).

Table 1. Androgenic plant genotypes of fully regenerated fertile androgenic plants.

Genotype of androgenic plant	Accession code of the genotypes	Number of fully developed fertile plants
Kurtovska kapija SR	8	3
Piran	MK1	2
Zlaten medal	7	2
Feherozon	15	5
Pritavit F1	1H	1
Majori	4H	1

The collected seeds were used for design of experiment for characterisation of the androgenic pepper lines that were obtained from the pepper genotypes used in the process of androgenesis. The experiment was established at the greenhouse of Faculty of Agriculture - Strumica. Firstly, the seeds from the androgenic plants of three pepper genotypes Kurtovska kapija (two androgenic lines), Piran (two androgenic lines) and Feherozon (three androgenic lines) were sowed in peat and perlite mixture in polyethylene containers (Figure 1a). The plantlets were transplanted into polyethylene pots, filled in with mixture of soil, peat and perlite. In the greenhouse, a drip system was established, so the plants were irrigated and fertilized in the same time. The pots were aligned according to randomized block design in order the growing and environmental conditions of the greenhouse to equilibrate (Figure 1b, c). The pepper androgenic lines used for characterisation are presented in Table 2.
Beside plants of pepper androgenic lines, there were planted also plants from the original mother plants used for androgenesis (control plants) (Table 2), so the characteristics of the androgenic lines were compared to the original pepper genotype used as anther donors. In total 40 plants of each androgenic line and 20 plants from original pepper genotype were used for characterisation. The reason for selection of Kurtovska kapija, Piran and Feherozon androgenic lines for further experiment and exclusion of the other three androgenic lines of genotypes Zlaten medal, Pritavit F1 and Majori is because these genotypes are of special interest for the Macedonian horticulture. Kurtovska kapija is genotype that traditionally is grown in the south eastern region of Macedonia for processing, and in the latest years there are different pests and diseases that attack this variety, so creation of new lines and their characterisation and testing in different conditions might bring out solutions. Piran is Macedonian pepper variety which is newly created, so is good to be tested for its biotechnological and agronomical potentials. Feherozon is Hungarian variety of bell pepper, but according to the literature with excellent androgenesis potential. All the facts stated above were important for the decision of future experimental work.

Different phenological and morphological parameters were observed in the androgenic pepper lines and control plants according to Descriptors for *Capsicum* spp. The phenological parameters that were observed are following:

- Sowing date
- Transplanting date
- Flowering (beginning and full flowering)
- Fruiting (beginning and full fruiting)
- Ripening (technological and botanical phase)

In different phenological phases of pepper plants development different morphological parameters were observed and measured as following:
- Plant height
- Plant stems width
- Internodes length
- Number of leaves
- Leaf height
- Leaf width
- Number of flowers

Fruits are most important organ of pepper plants because they provide the seeds for the next year experiment, so special attention was paid to fruits. Certain numbers of morphological traits were measured in technological and botanical fruit phase:

- Number of fruits in technological phase
- Number of fruits in botanical phase
- Number of fruits in technological phase per harvest
- Number of fruits in botanical phase per harvest
- Fruit length (cm)

- Fruit width (cm)
- Fruit weight (g)
- Fruit wall thickness (mm)
- Number of fruit locules
- Fruit dry matter content (%)
- Number of fruit seeds
- Dry weight of seeds per fruit (g)
- Dry weight of 1000 seeds

The values of some of the fruit morphological traits of control genotypes and androgenic lines are presented in Table 3a and Table 3b.

The seeds of different fruits of interest were collected separately, marked and stored in the gene bank of the Faculty of Agriculture - Strumica for the next year characterisation experiment of original mother plants and different pepper androgenic lines.
Beside the phonological and morphological parameters, samples of leaves and fruits in different plant growth and fruit ripening phases (technological and botanical) were collected in order to be analysed for the macronutrient and micronutrient content.

As it was reported in the First Interim Report, the experiment of androgenesis induction was conducted in the second experimental year, starting with thirteen pepper (*Capsicum annuum* L.) genotypes of different type and origin used as anther-donor plants in 2007. Seeds of thirteen genotypes of pepper (*Capsicum annuum* L.) were sowed in containers and 10 plantlets from each genotype were transplanted into polyethylene pots in May 2007 (Figure 2a). Regular cropping practices regarding fertilization and irrigation were practiced. Development of flower buds started during July 2007.

Since in the first experimental year, the anthers cultivated on MS medium did not show any embryogenic response e.g. there were no embryos emerging from the anthers (First Interim Report, Table 3,4), in the second year only the method of Dumax de Valux et al. (1981) was used for androgenesis induction and production of androgenic pepper plants. The same protocol as described in the First Interim Report was applied for induction of androgenesis in the experimental year 2007. Again, the method of Dumax de Valux et al. (1981) is explained below.

Certain number of anthers of each genotype was cultured on Cp inductive medium. Fifteen anthers were plated per 5 cm diameter Petri dishes, the concave side touching the Cp inductive medium (Dumas de Valux et al., 1981) supplemented with 0.01 $mg \cdot l^{-1}$ Kinetin and 0.01 $mg \cdot l^{-1}$ 2,4-D. The first 8 days the cultures were incubated at 35°C in darkness in order this stress treatment to induce androgenesis (Figure 2b). The following 4 days the anthers were transferred to Cp medium with the same composition but incubated at 25°C, with a photoperiod of 12 hours light at 30-40 $\mu mol\ m^{-2}\ s^{-1}$ (Figure 2c). After 12 days of induction in Cp medium anthers were transferred to R_1 medium (Dumas de Valux et al., 1981) supplemented with 0.1 $mg \cdot l^{-1}$ Kinetin and placed in a growing chamber at 25°C, with a photoperiod of 12 hours light at 30-40 $\mu mol\ m^{-2}\ s^{-1}$. The anthers were regularly observed and changes were noted. During the incubation of anthers, they were stained with acetocarmine, squashed on microscopic slides and observed under microscope if any changes are appearing on microspore level. Parameters as number of cultured anthers, number of emerged embryos, embryogenic anthers (%), number of embryos per 100 anthers, number of embryos regenerated in plants were observed (Table 4). The androgenic potential was determined from the percentage of embryogenic anthers according to Mitykó et al. (1995) classification.
Embryos emerging from the anthers were transferred into glass jars onto V_3 hormone-free medium (Dumas de Valux et al., 1981) for further development into plantlets and placed in the growing chamber at 25°C, with a photoperiod of 12 hours light. The plantlets obtained via the process of androgenesis from *in vitro* conditions were transplanted into sterile mixture of peat, perlite and sand (1:1:0.25) and placed in clime chamber for acclimatization.
In the second experimental year, from all thirteen genotypes involved in the process of androgenesis, only 2 responded with embryo formation, Tura and Majori, where Tura gave 5 embryos, but only 3 were regenerated in fully developed plants. Majori gave only one embryo which did not succeed to be regenerated in fully developed plant. The cultured anthers of the other 11 pepper genotypes developed either callus or they became black and deteriorate.
In May 2008 three fully regenerated plants of genotype Tura were transferred from the clime chamber to the greenhouse conditions for further observation and development of fruits and seeds.

In April 2008, the collected seeds from fruits with good characteristics during the experimental year 2007 (the pepper genotypes presented in Table 2, first generation of androgenic lines), were sowed in peat and perlite mixture, and after the plantlets were transplanted in the polyethylene pots filled in with soil, peat and perlite and placed in the greenhouse in the same manner as in the first experimental year. These plants are the second generation of androgenic lines. The purpose of repetition of the experiment is to compare the second generation of androgenic lines to the first one, and to the original genotype in order to see how much these androgenic lines are polymorphic, stable, similar to the original genotype or not. The same phonological and morphological parameters will be observed and noted, as well as seed collection from the second generation of androgenic plants will be performed. In this way, the agricultural biodiversity of the Faculty of Agriculture is enriching, giving more starting options for pepper selection.

In April 2008, seeds of 7 pepper genotypes were sown in polyethylene containers for production of mother anther-donor plants which will be used as anther-donors for induction of androgenesis during the third experimental year according to the same protocol of Dumas de Valux et al. (1981). Possible induction of embryogenesis and embryo regeneration into fully developed plants will give reproduction material (seeds) with unique characteristics that can be used for future characterization and breeding.

From July 2007 to June 2008 the return fellow, Mrs. Fidanka Trajkova, took participation at different trainings, meetings and forums about important issues in agriculture and science in Republic of Macedonia, travelling by bus/car.

From 04 to 05 October 2007 the return fellow, Mrs. Fidanka Trajkova, took participation at the Third Congress of Ecologists of the Republic of Macedonia with international participation, held in Struga.

From 15 to 19 June 2008, the return fellow, Mrs. Fidanka Trajkova paid visit to the Mediterranean Agronomic Institute of Bari, Bari, Italy, in compliance with her work.

References:

IPGRI, AVRDC and CATIE. (1995). Descriptors for *Capsicum* (*Capsicum* spp.). International Plant Genetic Recourse Institute, Rome, Italy; the Asian Vegetable Research and Development Center, Taipei, Taiwan, and the Centro Agronómico Tropical de Investigación y Enseñanza, Turrialba, Costa Rica.

Dumas de Valux R., Chambonnet D., Pochard E. (1981). *In vitro* culture of pepper (*Capsicum annuum* L.) Anthers: high rate plant production from different genotypes by + 35ºC treatments. Agronomie 1(10): 859-864.

Mitykó J., Andrásfalvy G., Csilléry G., Fári M. (1995). Anther culture response in different genotypes and F_1 hybrids of pepper (*Capsicum annuum* L.). Plant Breeding 114, 78-80.

Mitrev S., Trajkova F. (2007). First Interim Report, Implementation of Novel Biotechnological Methods Towards Food Security. Reintegration Grant of NATO Security Through Science Programme.


a

b

c

Figure 1.

a. Plantlets of different androgenic lines in the nursery, before transplantation to pots; b. Transplanted plants of the three controls and seven androgenic lines into pots and randomized block experiment design for characterization; c. Plants of the three controls and seven androgenic lines in full development and fruiting.


a

b

c

Figure 2.

a. Plantlets of different anther-donor genotypes just after transplantation in to pots; b. Anther culture in induction chamber on Cp medium at 35^{o}C, in darkness; c. Anther culture in growing chamber on Cp medium at 25^{o}C, 12 hours light.

Table 2. Mother genotypes and androgenic lines of pepper used for characterisation under greenhouse conditions.

Genotype of mother plant	Accession code of control genotypes and androgenic lines	Experimental code of control genotypes and androgenic lines	Number of plants for characterisation	Growing conditions
Kurtovska kapija SR	8	KKk	20	Greenhouse
Kurtovska kapija SR	2/8	KK1	40	Greenhouse
Kurtovska kapija SR	14/8	KK2	40	Greenhouse
Piran	MK1	Pk	20	Greenhouse
Piran	14/1 (1)	P3	40	Greenhouse
Piran	14/1 (2)	P4	40	Greenhouse
Feherozon	15	Fk	20	Greenhouse
Feherozon	7	F5	40	Greenhouse
Feherozon	6	F6	40	Greenhouse
Feherozon	5	F7	40	Greenhouse

Table 3a. Average of different morphological fruit characteristics in technological phase of fruit ripening.

Experimental code of pepper controls/androgenic lines	Plant length (cm)	Plant width (cm)	Total fruit weight (g)	Fruit weight without pedicel (g)	Fruit wall thickness (mm)	Number of locules	Dry seed weight (g)	Seeds number	Dry matter content (%)
KKk	9.888	4.711	48.688	40.089	0.39	2,2	0.853	170.6	9.42
KK1	10.123	5.031	60.336	51.53	0.369	2,0	0.927	165.08	8.12
KK2	11.216	5.266	70.890	61.338	1.894	2,0	1.186	166.75	7.86
Pk	30.336	6.743	75.902	62.018	0.5	4,4	1.416	258	11.44
P3	14.843	3.554	42.08	34.455	0.264	2,05	0.408	100.3	5.63
P4	15.638	3.543	43.879	36.193	0.254	2,05	0.427	102.45	5.64
Fk	8.89	5.3	63.515	54.583	0.392	3,1	0.599	138.1	4.62
F5	5.985	5.873	69.325	60.787	0.406	3,2	1.155	86.29	4.78
F6	8.444	5.349	71.072	62.475	0.386	2,85	0.422	100.18	5.07
F7	5.718	5.796	61.995	54.333	0.384	3,0	0.324	90.275	4.68

Table 3b. Average of different morphological fruit characteristics in botanical phase of fruit ripening.

Experimental code of pepper controls/androgenic lines	Plant length (cm)	Plant width (cm)	Total fruit weight (g)	Fruit weight without pedicel (g)	Fruit wall thickness (mm)	Number of locules	Dry seed weight (g)	Seeds number	Dry matter content (%)
KKk	10.656	4.965	58.385	48.915	0.326	2.0	1.429	216.4	8.56
KK1	10.912	5.461	73.34	62.519	0.379	2.0	1.321	208.05	8.62
KK2	11.371	5.592	79.678	67.67	0.354	2.05	1.607	198.25	7.92
Pk	13.544	3.374	32.169	26.322	0.21	2.1	0.774	138.4	8.98
P3	14.405	3.402	43.4	35.947	0.224	2.0	1.347	124.2	9.37
P4	14.696	3.822	47.999	39.220	0.221	2.05	1.144	126.45	9.31
Fk	8.457	6.289	101.822	90.772	0.565	3.4	0.978	173.8	7.0
F5	6.021	6.611	79.456	69.510	0.478	3.05	0.858	142.55	7.87
F6	9.541	5.337	68.936	62.362	0.427	2.8	0.585	102.35	8.16
F7	5.805	6.051	67.832	59.625	0.465	3.05	0.65	116.617	8.23

Table 4. Induction of androgenesis in different pepper (*Capsicum annuum* L.) genotypes according to the method of Dumas de Valux et al. (1981) in the experimental year 2007.

Accession code	Genotype	Number of cultured anthers	Number of emerging embryos	Embryogenic anthers (%)	Number of embryos per 100 anthers	Number of embryos regenerated in plants	Embryos regenerated in plants (%)	Embryogenic response
MK1	Piran	300	0	0	/	/	/	No
MK2	Kurtovska kapija BG	400	0	0	/	/	/	No
MK4	Zlaten medal ŠT	300	0	0	/	/	/	No
7	Zlaten medal SR	300	0	0	/	/	/	No
8	Kurtovska kapija SR	300	0	0	/	/	/	No
9	California wonder	300	0	0	/	/	/	No
15	Feherozon	335	0	0	/	/	/	No
1H	Pritavit F1	265	0	0	/	/	/	No
2H	Tomato shaped sweet	250	0	0	/	/	/	No
3H	Tura	410	5	1.57	3.35	3	60	Poor
4H	Majori	300	1	0.69	2.27	0	0	Poor
5H	Kincsem F1 HP14	300	0	0	/	/	/	No
6H	Vitamin F1 HPO13G	300	0	0	/	/	/	No

Table 5. Pepper genotypes used as anther-donor plants in the experimental year 2008

Accession code	Genotype	Country of origin
MK1	Piran	Macedonia
7	Zlaten medal SR	Macedonia
8	Kurtovska kapija	Macedonia
15	Feherozon	Hungary
1H	Pritavit F1	Hungary
3H	Tura	Hungary
4H	Majori	Hungary

4. Financial Reports

a) HOST INSTITUTION DIRECTOR

AWARD in Euro **11,000.00** ***PAYMENTS*** received *(specify currency)*: 2nd instalment 3,300.00 Euros

EXPENDITURE

Expenses related to the return project	**Currency** *(specify)*
Consumables	943.00 Euros
Services	740.00 Euros
Personnel	1,560.00 Euros
Equipment *(specify)* ……..……. ……. …….	
Other *(specify)*	

TOTAL EXPENDITURE 3,243.00

b) RETURN FELLOW

AWARD in Euro **14,000.00** ***PAYMENTS*** received *(specify currency)*: 2nd instalment 4,200.00 Euros

EXPENDITURE

			Currency *(specify)*
Personal Subsistence			3,320.00 Euros
Travel:	From/To	Period (from/to)	
	Travelling within the country	July 2007 - June 2008	204.00 Euros
	Strumica - Bari - Strumica	15 - 19/06/2008	512.00 Euros
Other			164.00 Euros

TOTAL EXPENDITURE 4,200.00

Both the Return Fellow and the Host Institution Director should indicate their agreement to this report by signing below.

Signature of Return Fellow: Signature of Host Institution Director:

Date: 04/07/2008 Date: 04/07/2008