IN VITRO INDUCED SALT TOLERANCE IN PEPPER *(CAPSICUM SP.)*

BY

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Abstract

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This study investigated the hypothesis that: The time of the transition of the young pepper plants into the saline environment is the critical stage, and *in vitro* culture can be used in future to ensure a sufficient lasting conditioning of the proliferated plants for a subsequent growth under saline conditions. Several *in vitro* culture protocols were tested to optimize regeneration, and to clarify to what extent it would be possible to condition pepper regenerants for a life under saline conditions. Results indicated that pepper plants were difficult to develop somatic embryos. Proliferation via axillary shoot was achieved using rooted shoots as explant in BA-free MS medium. Regarding salinity studies, wild species were more salt sensitive than cultivated pepper, and were genetically different as indicated by RAPD analysis. The salinity tolerance in pepper cultivars was mediated by a higher proline, higher activities of PRX and SOD and a lower uptake of Cl^-. It could be shown for the first time, that it is possible to adapt plants for the growth under saline conditions using *in vitro* treatments with NaCl (exponential increase of the NaCl concentration and 50 mM as a pre-treatment) with unrooted shoots as started explant material, but this effect was less when rooted shoots were used. It was possible to diminish the deleterious effects of NaCl with regard to plant height and survival rate until a certain time using pepper pre-treated with *in vitro* 50 mM NaCl, but this was a temporary effect, depending on the salt concentration, and was not observed during fruiting stage. The influence of the pre-treatment with 50 mM NaCl was stable enough to give a significant higher fruit yield at the end of the culture under non saline conditions which was repeatable using cv. ' Yolo Wonder'. *In vitro* pre-treatment with 50 mM NaCl increased proline content in the greenhouse-grown plants *ex vitro* treated with 200 mM in cv. 'De Cayenne'. However, K^+, Na^+, Cl^-, Ca^{++}, chlorophyll content and antioxidant enzymes were unchanged among different *in vitro* pre-treatments. Salinity tolerance mechanisms seems to be dependent on the plant stage.

Key Words	*In vitro* culture-Salinity tolerance- Conditioning- Epigenetic changes

6.a SUMMARY

Twenty three consecutive experiments were conducted in the tissue culture laboratory and glasshouses of Institute of Vegetable and Ornamental Crops, Department Plant Propagation, Erfurt, Germany during the period from 2001-2004. The conducted studies included the use of thirty nine different pepper genotypes, belong to six different species, in propagation and salinity experiments. Twenty three genotypes from Taiwan, seven genotypes were supplied from Gatersleben, Germany, five genotypes were kindly supplied from two seed Germany companies (Weigelt and Chrestensen), only 'Ace' was supplied from (Unwins company, UK). 'Shatta Balady' was obtained from two sources, the first one from Dr. Richter (Institut fuer Nutzpflanzenkunde, Witzenhausen, Germany) and the second one was obtained from local source, also, 'Marconi' was obtained from local source.

The aim of the present study is to clarify to what extent it would be possible to induce salt tolerance in pepper using *in vitro* propagation methods without changing the genotype. Our working hypothesis is the time of the transition of the young plant into the saline environment is the critical stage for the survival under saline conditions. *In vitro* methods can be used in future for the mass propagation of pepper and ensure a sufficient lasting conditioning of the regenerating plants for a subsequent growth under saline conditions.

This study was conducted with the following sub-aims: a) establishment and optimisation of *in vitro* culture methods of pepper (somatic embryogenesis, axillary shoot proliferation) for subsequent growth under saline conditions, b) determination of the salt sensitivity of cultivated and wild species of pepper, examination of the genetic differences, physiological and biochemical evaluation of salt tolerance. c) examination of the possibility to induce salt tolerance using *in vitro* propagation methods and verification of the transferability of the obtained results to other cultivars and species, d) examination of the stability of the induced salt tolerance in the greenhouse, e) proof of the induction character of the salt tolerance by obtaining knowledge about possible physiological and biochemical mechanisms as well as checking the genetic identity of the proliferated plants.

The salient findings of the present study are as follows:

6.1 Establishment and optimisation of *in vitro* culture methods of pepper.

6.1.1 Propagation via somatic embryogenesis.

1- Repeating the protocol of Buyukalaka and Mavituna with different type of callus did not allow the production of any somatic embryo.

2- Suspension cultures could be established and showed an increasing PCV using 4%. The number of globular-like structures increased using cell suspensions without sieving in presence of tri-potassium citrate.

3- Addition of 2 g/l activated charcoal to the medium increased globular like-structures, whereas the higher activated charcoal concentrations decreased their number. Also, G.-like structures increased with cell density increase up to 4% as a started cell inoculums using full MS.

4- We could not confirm the embryogenic nature of the globular-like structure using histological examination. Therefore, propagation via axillary shoot culture has been studied.

6.1.2 *In vitro* propagation via shoot proliferation.

1- 10 mg/l BAP gave around only one shoot/explant, and 0.5 mg/l NAA gave higher rooting and more healthy plantlets afterwards.

2- Increasing BAP to 50 mg/l did not significantly increased the shoot number. However, the subsequent rooting decreased with increasing BAP concentrations.

3- Using rooted shoots instead of shoots with removed roots increased number of axillary shoots/plantlet (3.5 and 1.333 respectively in cv. 'Shatta Balady') under lower BAP concentration (0.0 mg/l). The subsequent rooting occurred at higher level when rooted shoots interacted with 0.2 mg/l IBA, but, shoots with removed roots responded more under higher IBA concentration (0.8 mg/l).

4- MS medium produced significant higher axillary shoots compared to U medium.

5- Application of proliferation protocol to four different genotypes, resulted in a successful axillary shoot proliferation, but at different rates. Hot pepper genotypes responded more than sweet pepper genotypes.

6 .2 SALINITY STUDY

6.2.1 Determination of the sensitivity, examination of the genetic differences, physiological and biochemical evaluation of salt tolerance of cultivated and wild species of pepper.

1- Phenotypic parameters decreased with increasing NaCl concentrations in all pepper genotypes. The reduction in all parameters related to control was lower in cv. 'De Cayenne' followed by "Yolo Wonder", then 'California Wonder'. However, higher reduction % was in cv. 'Shatta Balady (2)'.

2- Using lower NaCl concentrations up to 200 mM indicated that no real germination has occurred under NaCl higher than 100 mM NaCl.

3- Wild species are genetically different and they were more salt sensitive compared to cultivated cultivars.

4- NaCl concentrations up to 50 mM improved the normal growth of seedlings.

5- The significant higher proline content was found in more salt tolerant genotype. However, a significant higher chloride concentration in the shoots was detected in the more sensitive one.

6- In cv. 'New Mexico 6-4' (cultivated cultivar) the PRX activity decreased under favourable salt concentrations and increased under higher concentration, however, in *'C. microcarpum'* (wild specie) the activity of PRX decreased with increasing NaCl concentrations.

7- The SOD activity increased with increasing NaCl in cv. 'New Mexico 6-4' (cultivated) especially in bands number 4 and 5 which were absent in wild specie and the band number 3 was absent in cultivated cultivar, however, the activity of SOD was unchanged in *'C. microcarpum'* (wild).

6.2.2 Examination of the possibility to induce salt tolerance using *in vitro* propagation methods and verification of the transferability of the obtained results to other cultivars and species

1- Application of ABA or JA did not improve the salinity tolerance in terms of shoot length in cv. 'De Cayenne'. However, the pre-adaptation with lower NaCl concentration (50 mM NaCl) increased shoot length significantly compared to other pretreatments (0.0 and 100 mM NaCl).

2- Application of exponential NaCl increase lowered the reduction in shoot length to non-significant level in cv. 'Shatta Balady' under 200 mM NaCl as a main treatment compared to control without salt using unrooted shoots as a started explant types. Also, % succulence significantly increased under exponential NaCl increase, compared to other procedures (liner and logarithmic NaCl increase) with shoot tips as explant type in cv. 'Shatta Balady'.

3- 50 mM NaCl as a pre-treatment in most cases showed a higher morphological parameters compared to other pretreatments (0.0 and 100 mM NaCl) using unrooted shoots as explant types.

4- The favourable effect of the exponential NaCl increase and the pre-treatment with 50 mM was unfavourable using rooted axillary shoots (weak effect with regard to leaf number).

5- The best time for application of the pre-treatment with lower NaCl concentration was after axillary shoot proliferation stage, because, our results showed that axillary shoot number and subsequent rooting decreased when the pre-treatment with 50 mM NaCl was involved during the proliferation stage.

6- The application of exponential NaCl increase to nine different pepper genotypes showed different responses using rooted shoots as explant type. Five genotypes were significantly responded to this procedure, however, four genotypes did not respond in terms of shoot length related to control.

7- Using rooted shoots as explant type decreased the deleterious effect of salinity compared to the shoots with removed roots.

6.2.3 Examination of the stability of the *in vitro* induced salt tolerance under greenhouse.

1-The phenotypic parameters decreased with increasing salinity, but it is possible to diminish the deleterious effects of NaCl with regard to plant height and survival rate until a certain time using a pre-treatment with 50 mM NaCl in the first experiment, but this was a temporary effect, depending on the salt concentration and was not observed with regard to fruit yield.

2-The influence of a pre-treatment with 50 mM NaCl was stable enough to give a significant higher fruit yield at the end of the culture under non saline conditions which was repeatable using Yolo Wonder in the first two experiments.

6.2.4 Proof of the induction character of the salt tolerance by obtaining knowledge about possible physiological and biochemical mechanisms as well as checking the genetic identity of the proliferated plants.

1- K^+ and chlorophyll content decreased with increasing salinity, whereas, Na^+, Cl^-, Ca^{++} and proline level increased. This deviation was cultivar dependent.

2- *In vitro* pre-treatment with 50 mM NaCl, in most cases showed a higher proline compared to other pretreatments (0.0 and 100 mM NaCl) using unrooted shoots as explant types under *in vitro* main treatment with salt in both cultivars. However, the same pre-treatment showed a significant higher proline content in cv. 'De Cayenne' under *ex vitro* main treatment with 200 mM NaCl comparing to other *in vitro* pretreatments.

3-The *in vitro* salinity tolerance mechanisms seems to be dependent on the:-

plant stage: with early plantlet stage, the tolerance is mediated by a relative proline level increase and a lower uptake of Na^+ and Cl^-, however, with old plantlet stage the tolerance is mediated by the maintenance of K^+ selectivity connected with increased Na^+ and Cl^- levels.

Rooted shoots: the tolerance is mediated by maintenance of K^+ selectivity connected with increased Na^+ and Cl^- in both stages and higher proline level only in early plantlet stage

4-The mineral concentration (especially NO_3-N and K^+) and EC in the soil substrate (peat substrate) increased with salinity, however, the behaviour of the pH was dependent on the conditions.

5-The activity of SOD increased with increasing NaCl concentration, but there was no change in the activity of PRX.

6-Using 10 different primers, no genetic differences could be found between pre and treated plants with salt within the genotype, the difference was only between the two tested genotypes, using one primer.

6.b Conclusion

1- Still difficult to propagate pepper *in vitro* with high proliferation rate. It is clear from our results that, different *in vitro* culture methods were only useful for breeding procedure, not for horticulture means.

2- The epigenetic adaptation to salt stress applying pre-treatment with lower NaCl (50 mM) and exponential NaCl increase was achieved only for vegetative growth not for fruit yield.

3- The response to salinity is dependent on the genotype and plant stage

4- There is a relation of integration between shoots and roots for salt tolerance.

5- The response of SOD was stable and the activity of SOD is considered one part of the stress response, however, the response of PRX was dependent on the environmental conditions, i.e; *in vitro* and *ex vitro*.

6- Stress tolerance seems to be partially mediated by:

- Maintenance of K selectivity and a higher proline level.
- Higher activity of SOD.

6.c Outlook

Propagation:

1- It is necessary to establish a repeatable protocol for somatic embryogensis in pepper.

2- Optimization of axillary shoot proliferation of pepper using rooted shoots.

3- Examination of the possibility to propagate pepper by cutting.

Salinity

Because the breeding for salt tolerance depends on a profound understanding of the physiology, genetics and molecular biology of plants under salt tolerant, more experiments are necessary with regard to:-

1- Screening for salt tolerance using higher number of wild species (Wild Germplasm) under stable and suitable conditions.

2- In the first stage (*in vitro* plants) our results revealed that, the salinity mechanisms depend on the plant stage i.e. with young stage mediated by proline, however, with old stage mediated by maintenance of K selectivity, therefore, evaluation of salt tolerance in details is important in pepper.

3- The plants appeared, under salinity, with different phenotypes, therefore, it is possible to use the simple selection procedure for inducing salt tolerance.