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# Proline's Function in Cold Stress and Osmoregulation in Carrot Tissue Culture Suspensions

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## Abstract

Wild type and high free proline suspension-cultured carrot cell lines were used to determine whether proline influences cold tolerance. In addition, various stressful environments were imposed on the suspensions by varying media ingredients, such as the addition of mannitol and abscisic acid (ABA), in an effort to induce proline production.

Results showed that an optimum concentration of proline hardened the control lines and acted as a growth stimulant under normal growth conditions. When both lines were grown at 3 C the high proline mutant line C-63, proved to be the hardiest. When grown at room temperature, C-63 was actually the weaker of the two lines. This relationship indicates that proline directly or indirectly influences cold hardness in carrot cells.

Mannitol, which induces osmotic pressure on plant cells, did not significantly increase proline accumulation. Abscisic acid had a lethal effect on the cells even at  $10^{-4}$  M. concentrations. Smaller concentration may still induce proline production, but this possibility was not explored.

## Introduction

A frequent reaction of plants exposed to stressful conditions such as dehydration or cold is the production of the amino acid proline (van Swaaij et al 1985). Likewise, observations in several plants indicate that free proline may protect the plants from stress. This relationship has repeatedly been recorded, but whether the high free proline counteracts the detrimental effects of the stress remains a controversy.

Tissue cultures provide a system to study the biochemical and physiological aspects of cold tolerance and osmoregularity in a controlled environment (Duncan). Carrot tissue cultures are specifically advantageous in this type of experimentation for several reasons:

1. The cells grow relatively quickly
2. High free proline mutant lines are available for experimentation, and
3. Carrot cells lend a particularly uniform method of transfer with a wide mouth pipette.

The specific lines selected for this experiment were TC-1, a wild type started in 1967, and the mutant line C-63, which was selected for its abnormally high proline accumulation in 1981.

## Materials and Methods

The carrot cell lines TC-1 and its high proline mutant line C-63 were maintained on Murashige and Skoog MX medium (Murashige and Skoog 1962) in 125 ml. Erlenmeyer flasks. The flasks were kept at 28 C on a reciprocating shaker and sub-cultured every seven days. A preliminary check was performed on TC-1 and C-63 for tolerance to the amino acid analog azetidine-2-carboxylate. Toxic amino acid analogs are used to inhibit cell growth. The corresponding natural free amino acid will reverse the growth inhibition of the analog. Thus cells that accumulate abnormally high levels of proline will be resistant to growth inhibition of the A2C analog. (Widholm 1980). Analogs are compounds that closely resemble the normal amino acid in structure and function but differ enough to interfere with the proper action of the amino acid or product made from it (Widholm 1984). Since C-63 is a high proline mutant, it should by nature be resistant to the proline analog. Since the two lines TC-1 and C-63 were selected in 1967 and 1981 respectively, mutations may have occurred over time. Testing for A2C tolerance assures that the C-63 cell line is still a high proline mutant. Concentrations of A2C used were  $10^{-4}$ ,  $10^{-3}$ ,

and  $10^{-2}$  M. As expected, all of the above concentrations of A2C were lethal to the wild type TC-1, but C-63 the high proline mutant line, was resistant to concentrations of  $10^{-4}$  and  $10^{-3}$  M, which are high enough concentrations to indicate the lines have not mutated with respect to proline levels since their original selection.

In order to understand the effects of exogenously applied proline on carrot tissue cultures at normal environmental conditions, and to find an appropriate concentration with which to work with under cold conditions, preliminary experiments were performed in which proline concentrations of 10, 20, 30, 40 and 50 mM concentrations were added to the MX media. Again, cells were grown at 28C for two weeks. Fresh weights were measured by vacuum filtering the cells on Mira cloth.

In addition to chemically imposing stress, proline accumulation in both lines was measured as a function of an environmental change. Both the wild type and the high proline mutant carrot cell lines were grown at 28C and 3C on MX and high proline media. In the first part of this experiment both lines were grown on general MX media. Half the flasks were grown for ten days at 28C while the remaining half were exposed to the cold (3C) for an eight day period after which they were grown at 28C as the control group for ten days. In the second part, both lines were grown on a high proline media (50mM). Half the set was grown at 28C for seven days while the remainder of the flasks were kept at 3C for six days and allowed to grow at 28C afterward for seven days. Thus, in each set, the amount of time the cells were allowed to grow at room temperature was kept constant with the only difference between the control and the experimental groups being the cold period. It was expected that the cold environment would halt growth with or without the addition of proline. Those cells grown on a high proline media and the high proline mutant were expected to grow more rapidly once they were returned to a normal environment.

In order to induce osmotic pressure on the carrot cells, both lines were tested on media with mannitol of concentrations of 250 and 500 mM. Cells were harvested after two weeks of growth at 28C after which they were dried on Mira cloth and weighed. Proline was extracted and assayed using ninhydrin reagent. An optical density of each final product was read and compared with a standard based on various known proline concentrations (Aust. 1973). Proline was assayed per one gram fresh weight of cells. In some cases where it was impossible to harvest one gram, even from three flasks 150 ml., an amount less than one gram was assayed, unless the final fresh weight was less than .40 g. Mannitol is a sugar alcohol, which increases the solute concentration of the media, dehydrating the cells. By simulating dehydration it was expected that the cells would produce proline in response to the stress.

Proline accumulation is normally associated with senescence (Chu et al 1978). Since the hormone abscisic acid is also associated with this stage of growth, cells were also tested on ABA concentrations of  $10^{-5}$  and  $10^{-4}$  M. It was expected that ABA would likewise induce a chemical stress on the carrot tissue cultures and thus increase proline accumulation. The cell suspensions were grown according to the procedure described with mannitol.

## Results

The carrot tissue cultures showed no significant mutation related to proline content in either cell line according to the preliminary test results with A2C. Accordingly, the high proline line was resistant to the proline analog of concentrations up to the  $10^{-2}$  M and the TC-1 was not.

As expected, the high proline mutant cell line (C-63) survived the cold stress considerably better than did the control line (TC-1). The increase in weight of the mutant line exposed to cold which was later allowed to grow under normal conditions was 2.99g per 50ml. Media as opposed to .40g of the control line. When the two lines were grown on media including 50mM proline, the control line TC-1 showed significant improvement in weight increase under both normal and cold conditions. Without exogenously applied proline at room temperature, TC-1 increased in fresh weight by only .65g. The difference in weight increase without proline and with proline at 3C is between .40g and 1.71g respectively. This difference indicates that the exogenous proline aided

in shielding against cold stress and also acted as a growth stimulant at a normal growth temperature.

Preliminary test data with the two concentrations of mannitol does not show that the osmotic stress imposed by mannitol contributes significantly to proline accumulation in the control lines. The difference in the amount of proline accumulation between TC-1 cells grown on mannitol media and those grown on the MX media was only an increase of .001g. A more significant increase in proline accumulation due to mannitol was found with the mutant cell line; proline weight increased .19g as a result of the osmotic pressure. The higher concentration of mannitol (500mM) proved lethal.

Both concentrations of ABA proved to be lethal. When it was found that the suspensions were not showing signs of growth, as in the case of the ABA media and the 500mM concentrations of mannitol, the media was extracted from the flask and substituted with MX media. This substitution was done in order to understand whether the two harmful chemicals caused a strain, which was reversible (elastic) or permanent (plastic). Since none of the suspensions showed any improvement after two weeks of growth with MX media, the toxic effect of ABA and the 500mM concentration of mannitol were plastic.

The preliminary proline experiments served to find a level of proline most conducive to cell growth. It was expected that if the fresh weight was graphed against the proline assay, a correlation resembling a bell curve would result. For the high proline line this expectation was precisely the case: an assay weight of .19mg. proline was the optimal level conducive to cell growth according to fresh weight. For the control line, the relationship was not as clear cut. In this case an assay weight lower than .002mg. was optimal. Whether an assay weight lower than .002mg. proline would yield an even higher fresh weight is not known.

## Discussion

Results indicate that proline does perform a function in cold tolerance among the carrot tissue cultures tested, but only within certain limits. This knowledge can possibly be used as a significant tool in plant breeding programs. If direct relationships are consistently found among similar experiments, a plant breeder looking for strains particularly tolerant to cold could possibly do so by measuring proline content of those lines. On a different tangent, since added proline acted as a growth stimulant in the control line at room temperature, carrot cell lines could actually be induced to grow under controlled environmental conditions by inducing regulated cold shock treatments. Further experimentation is needed in order to find an appropriate temperature as well as an ideal proline concentration.

No significant correlations can be made between osmotic stresses placed upon the cells by the mannitol. As for the added ABA, further trials are needed to find an appropriate concentration that would allow the cells to still survive in order to see whether proline accumulation would eventually occur.

## Future Prospects

Several variables leave specific correlations unresolved in this experiment. The following are possibilities for continuing research dealing with proline accumulation

1. Although one of the purposes of the preliminary experiments was to find appropriate concentrations of mannitol, ABA, and proline, further tests with an increased range of these chemicals could finer correlate their specific effects.
2. The above chemicals added to MX media could be combined to add to the variables.
3. Sodium chloride could be used in order to test the two carrot lines' reactions to dehydration.
4. It has been suggested in previous experimentation that the accumulation of proline is a consequence of a specific metabolic event occurring at a critical temperature rather than the

result of a continuous spectrum of temperature affected changes. For this reason the amount of time the carrot tissue cultures were exposed to the cold as well as the amount of time they were allowed to recover could be further varied.

5. In addition to cold tolerance, the cell lines could be tested for frost tolerance as a function of proline accumulation.
6. Cell cultures are not so simple that only one amino acid could be assumed to be involved in the physiological improvement of stress tolerance. Although proline is the amino acid accumulated most abundantly in water stressed plants, other amino acids such as asparagine, amide, and glutamine may also be involved (Stewart and Larher 1980). Other amino acids could be analyzed in conjunction with proline as a function of cold tolerance.

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