A PIXE study of vitrification of carnation in vitro culture

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Abstract

PIXE (Proton Induced X-ray Emission) is a well-known method for elemental analyses of specimens in applied studies. In this paper, we report results of an application of PIXE in trace-element analysis of normal and vitrified carnations in vitro culture. Experiments were performed to study the vitrification in connection with the trace elements in carnation tissues. About two hundred PIXE spectra were obtained from seventy samples with an irradiation of 3 MeV protons from the NEC 9SDH-2 Pelletron tandem accelerator. From the PIXE analysis we determined the trace element composition of normal and vitrified carnations. Our results indicate that there is a significant change of K, Ca, Fe and Zn contents in the vitrification process.

1. Introduction

The technique of plant cell and tissue culture, which has been widely used in agriculture to increase productivity, plays a central role in the rapid growth of plant genetic engineering. However, for plantlets cultured in vitro, a malformation problem called vitrification or glassiness [1] often appears. The vitrification phenomenon resulting in the occurrence of numerous short stems and less chlorophyllous leaves has been found in many kinds of plants [2]. As is well-known, vitrified species survive poorly as the environmental condition is changed from "in vitro" to soil in the green house. The failure of transferring vitrified plants is often attributed to malfunctioning [3–5]. And earlier investigations [6–11] have shown that many factors affect the vitrification, arising from the culture medium such as the agar concentration [6,7], the cytokinin type and concentration [8], the water uptake [1], and the evolution of the pH content [9,10]. It has also been found that the plant culture in low temperatures can increase the rate of normal shoot [11]. Phan and Hegedus [2] investigated the enzyme activity in both the normal and vitrified apple-plants, and reported a lower activity of enzymes found in the vitrified plants. Paques and Boxus [9] made an investigation of the vitrified apple-plants and showed that vitrification is related to the nutrient medium composition. However, the actual causes of physiological disorders associated with vitrification remain still to be understood.

Trace elements may plan an important role in the growth cycle process of plants. For example, manganese and iron are known as elements that play somewhat important roles

in photo-synthesis [12]. Calcium ions have been implicated in the regulation of numerous physiological processes [11]. Potassium ions, even at a very low level, can greatly affect the tissue appearance and lead to alternations of plant tissue metabolism [6]. However, little information is available on the possible relation between vitrification and mineral or organic compounds in the culture medium. Scarcely investigation has been undertaken on the possible relation between vitrification and trace element contents of cultured plant tissues. In the present work, we attempt to explore this relation in a detailed PIXE (Proton Induced X-ray emission) analysis.

PIXE is known as a very sensitive, multi-element, and simultaneous analytical technique for accurate determinations of elemental compositions in quantities of the order of ppm. And carnation is known as a plant species with a high probability of occurring vitrification phenomena in culture processes. In this work, we use the external-beam PIXE method in conjunction with the thin target and internal standard method to carry out PIXE spectra measurements for samples of carnation seedings cultured in vitro, and analyze the trace element compositions for normal and vitrified carnation tissues.

2. Experiment

2.1. Culture environment and target preparation

We cultivated carnation seedings in the Murashige and Skoog (MS) medium [14,15] in vitro supplemented with auxin containing 0.2 ppm IAA (indoleacetic acid) and various concentration of 6-BA (6-benzylaminopurin) as the cytokinin. Normal and vitrified carnation plantlets can be

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distinguished from their appearances. Altogether, seventy samples have been obtained from the culture prepared as follows:

A) For a comparison of the differences of the trace element composition between normal and vitrified carnation tissues, three different kinds of normal and vitrified carnation plantlets were cultured. They are classified as group I, II and III. For the first two groups, normal and abnormal carnations were cultured in the same medium (kept at constant temperature 25–28°C and light illumination) but in different vitro; while for the third group, normal and vitrified carnations were cultured in the same vitro.

B) For investigation of the low temperature effect, both the normal and abnormal carnation tissues were cultured for 20 days using same carnation species in the MS medium with 0.2 ppm IAA and 0.5 ppm 6-BA, and in eight small bottles to form four groups. These small bottles were put in a thermostat of 10°C for cold treatment. The cold temperature was maintained for four groups, respectively, for a time period of 4, 8, 12 and 16 days, followed by a continuing culture at a constant temperature of 25–28°C for 16, 12, 8 and 4 days. A time period of 20 days was kept for the culture of all groups.

C) For the study of the influence of auxin with different 6-BA concentration on vitrification, thirty samples for normal and abnormal carnations were prepared. They were cultivated for two weeks in the MS by adding 0.2 ppm IAA and different 6-BA concentrations from 0.1 to 1.5 ppm in steps of 0.1 ppm. A total of fifteen groups was used for the experiment.

D) For the study of the effect of active and genetic substances on vitrification, samples of four groups of vitrified carnation tissues were prepared. They were cultured in four small bottles under the same condition as the medium for two weeks. Two of four groups were added a small amount of juice extracted from normal carnation leaves, while the other two not.

All normal and vitrified carnation plantlets so cultured were then taken out from the medium. After cutting off polluted parts, the residue was rinsed with purified water and then dried and carefully weighed. Later it was transferred to a quartz vial and put in a low-temperature oxygen plasma oven for ashing. In order to improve the sensitivity, samples were pre-concentrated using a dry-ashing procedure. The residual ash was dissolved with nitric acid (2H HNO_3) and an appropriate amount of yttrium was added as an internal standard. Droplet of the obtained solution was then spotted on to a thin Mylar backing (3 μm thickness) to form a circular target spot with a size of less than 3 mm in diameter. After dried at room temperature, the target was then ready for PIXE measurement. During the experiment, the beam spot on the target was kept to cover the entire area of sample. Three targets were made with one sample solution, and each target was used in the measurement.

2.2. Analysis and calibration of the system

A sketch of the experimental arrangement is shown in Fig. 1. The proton beam of 3 MeV was produced from the NEC 9SDH-2 pelletron tandem accelerator located at the Institute of Physics, Academia Sinica in Taipei. The X-ray measurements were performed with an external-beam milliprobe [16] using a Canberra 30 $mm^2 \times 5$ mm Si(Li) detector, placed at 135° relative to the incident beam. A Kapton foil of 10 μm in thickness was used as the exit window through which the beam passes into the open air. And a funny absorber made of a 640 μm Mylar foil with a small hole of 0.65 mm in diameter at the center was placed in front of the detector to attenuate the intense flux of light element X-rays.

All spectra were recorded with conventional electronics followed by a Canberra series-95 MCA. The data were analyzed to determine amounts of trace element concentration. The elemental concentration of one sample was taken from data after averaging over measurements of three targets. In the analysis, we have used the GUPIX 94 software package [19] for evaluating the areas of the X-ray peaks. Also, we used the internal standard method for determination of elemental concentration. A known amount of a non-interfering element yttrium (Y) was added to the sample as internal standard (see Section 2.1) so that elemental concentrations of the sample can be
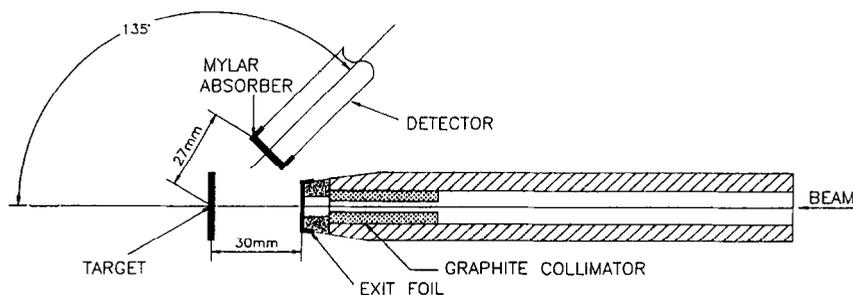
Fig. 1. Experimental setup for the external-beam PIXE measurement.
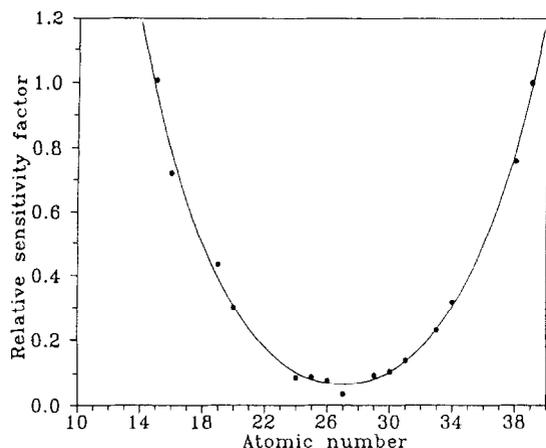
Fig. 2. A plot of relative sensitivity factor η as the function of atomic number Z, obtained for the detection system used in the present PIXE measurements.

determined relative to the concentration of the standard, using the formula

$$C_i(\text{ppm}) = \eta N_i C_y(\text{ppm})/Y_i,$$

where N_i and Y_i are, respectively, the numbers of counts under the peak area for elements contained in the analyzed target and the internal standard, C_y the known concentration of the internal standard element, and η a sensitivity factor of an element relative to the internal standard element, which can be measured using standard reference materials and internal standard. For this purpose, a thin target of multi-element mixture (including elements between P and Y for $Z = 15$–39) was prepared and used for the calibration measurement to determine the target sensitivity factor as the function of atomic number Z. The results are shown in Fig. 2, where black points are the η-values for various elements normalized to yttrium ($\eta = 1.0$ for Y). The smooth curve is the result of a least-squares fit to the data and was used for qualitative calculations of elemental concentration. As a check, the detection system was used to make a PIXE analysis of a

Table 1
Results of the measurement of contents of standard reference material 1577b

Element	Certified value	This work
P	1.10±0.03 wt.%	0.92±0.10 wt.%
K	0.994±0.002 wt.%	1.00±0.08 wt.%
Ca	116±4 ppm	126.3±0.71 ppm
Mn	10.5±1.7 ppm	7.4±1.3 ppm
Fe	184±15 ppm	178.0±5.6 ppm
Cu	160±8 ppm	152.8±4.4 ppm
Zn	127±16 ppm	129.5±1.1 ppm
Rb	13.7±1.1 ppm	12.7±1.7 ppm

standard reference material 1577b (Bovine Liver, purchased from National Institute of Standards and Technology, USA) to determine the contents of elements. The results are listed in Table 1 where a comparison between elemental concentrations of certified values and the measurements is given. The good agreement indicates the reliability of the obtained relative sensitivity factor for our detection system and hence the reliability of our PIXE analysis data.

3. Results and discussion

We have measured a total of about 200 PIXE spectra from 70 samples. To illustrate results of the measurements we show in Fig. 3 typical PIXE spectra obtained from a 3 MeV proton beam bombardment on two samples of normal and vitrified carnations. From the analysis of PIXE spectra we obtained the contents of K, Ca, Mn, Fe, and Zn in the examined samples. Tables 2 to 5 summarize the
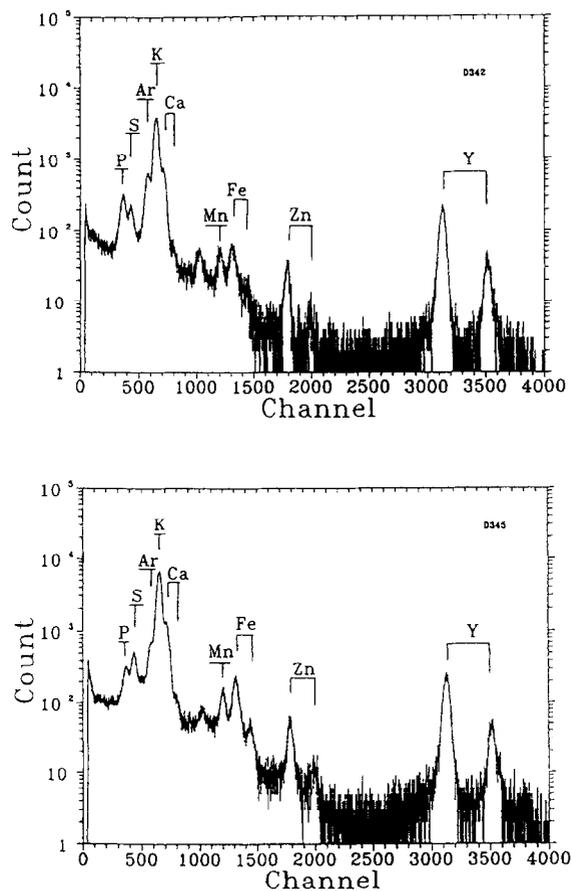
Fig. 3. Typical spectra for a 3 MeV proton beam incident on two samples (D342 and D345) of carnations prepared as described in the text; top: normal carnation; bottom: vitrified carnation.

Table 2
Elemental content in ppm of normal (N) and vitrified (V) carnations obtained from the PIXE analysis

Element	Group I		Group II		Group III	
	N	V	N	V	N	V
K	57520±960	75530±6500	66150±510	77460±390	48660±1100	75410±6400
Ca	1915±7.0	2457±88	1921±73	2312±450	1101±65	2641±210
Mn	109±0.17	111.1±3.6	116.8±4.4	113.3±0.97	82.6±2.8	211.9±5.8
Fe	317.5±7.5	661.0±2.0	329.2±8.4	669±33	78.1±8.4	299.3±3.9
Zn	121.8±0.25	184.3±6.5	119.4±0.72	175.1±1.9	93.7±1.0	117.0±4.2

Table 3
Contents of trace elements of vitrified (A) and normal (B) carnations after cold treatments

Sample	Elemental content [ppm]			
	Ca	Mn	Fe	Zn
4A	2887±81	403±25	313±21	108.2±2.9
4B	1834±110	132.3±5.3	123.3±5.3	82.9±3.1
16A	3164±140	363.0±1.1	514±29	158.4±3.8
16B	2506±290	379±29	571±32	172.8±6.0

Table 4
Growth rate of normal carnation seedings at different 6-BA concentrations

6-BA [ppm]	Normal seedings [%]
0.3	99
0.5	83
1.0	60
2.0	50

results where the values shown are the mean concentrations obtained in two or three measurements. A summary of the results now follows:

3.1. Comparison of concentration of trace element between normal and vitrified carnations

Table 2 shows measured contents of elements K, Ca, Mn, Fe and Zn in groups I, II and III for normal and vitrified carnations. It is noted that there appears a significant difference of elemental contents between the normal and vitrified carnations in all three groups. The vitrified ones were found to have considerably higher contents of

K, Ca, Mn, Fe and Zn (except Mn for group II) as compared to the normal ones. This indicates clearly that the vitrification is closely related to these trace elements in plant tissues. It is likely that the disorder of metabolism occurring in the vitrified plant leads to the disorder of absorption. In an earlier work by Paques and Boxus [9] vitrified apples after being cultured for 20 days were found to become heavier by a factor of 1.5 to 3.0. They suggested an enhancement of the vitrified apple metabolism. Our results which show a similar increase of the trace element contents, support their hypothesis of enhanced metabolism in vitrified plants.

3.2. Low temperature effect

We have measured the elemental contents in eight samples divided into four groups. These groups belong to same species of carnations cultured in the same condition but treated by cold temperature for different periods of time. Samples A and B were prepared, respectively, for the vitrified and normal carnations. Table 3 lists our results on the cold treatment for 4 and 16 days. The numbers affix to A and B show the time periods (day) of the cold treatment. We found that the cold treatment leads gradually to a change of Ca, Mn, Fe and Zn contents. It is noted that samples 4A and 4B with cold treatment for a period of four days appear to induce a clear difference of Ca, Mn, Fe and Zn contents. As the cold treatment is continued for a period of 16 days, the trace element concentrations for samples 16A and 16B become about the same. This indicates that cold treatments with a longer period of time may have a curing effect on vitrification. Such curing effects have been found in previous works [2,12], although it was reported that the curing effect of cold treatment is

Table 5
Effect of the active and genetic substance on vitrification

Groups of vitrified Carnation	Elemental content [ppm][a]			
	Ca	Mn	Fe	Zn
With extracted juice	1475±1.0	125.4±2.3	67.8±5.2	75.2±4.5
Without extracted juice	2685±300	167.5±8.9	148.7±1.8	89.0±3.4

[a] Obtained by taking an average of data of two groups.

short-lived, and the second generation of "cured" plants tends to become vitrious again.

3.3. The effect of auxin

Auxin, a substance very important to plant growth, is often used in the plant cultures. The cytokinin type and concentration of the auxin have been adopted and discussed in a number of previous works [8,9]. Here, we have used IAA and 6-BA to supplement the plant cell and tissue for the growth. With the adding 0.2 ppm IAA with various 6-BA concentrations to the culture medium, the culture was performed for two weeks. Thirty samples of normal and vitrified carnations were then obtained to examine the growth rate of cultured plantlets. Table 4 lists the results for normal carnation using a 6-BA concentration of 0.3, 0.5, 1.0 and 2.0 ppm. We found that the normal carnation has a higher growth rate at 0.3 ppm 6-BA concentration.

We also used various 6-BA concentrations from 0.1 to 1.5 ppm in the culture to examine the elemental absorption of carnation tissues for both the normal and vitrified ones. Results are shown in Fig. 4, which indicates that at 6-BA concentration lower than 0.5 ppm both the normal and vitrified carnations have about the same tendency to absorb Ca, Mn, Fe and Zn. while at 6-BA concentration higher than 0.5 ppm the absorption of these elements seems to become randomly. This reflects possibly a disorder of metabolism in the plant culture process at high 6-BA concentration.

3.4. The effect of an active and genetic substance

As is generally believed, the normal plant leaf contains an active and genetic substance [18]. To investigate the effect of an active and genetic substance, we have extracted a small amount of juice from the normal carnation leaves and added it to the medium used in the culture for two weeks. Four groups of vitrified carnations were investigated: two groups with adding extracted juice while the other two groups without. Results of elemental analysis of these four groups are given in Table 5. It is noted that the groups with added juice of normal leaves have considerably lower contents of Ca, Mn, Fe and Zn. And the cultured plantlets for the groups with adding juice showed a normal appearance. This indicates that the active and genetic substance has a curing effect, and tends to turn the vitrified tissues into normal. However, the vitrification phenomenon may reappear again in the normal ones, as the culture in the medium was continued for three weeks or longer. Our hyperthesis to explain this is that the vitrified plantlets have a lack of the active and genetic substance which can not be compounded by themselves. Thus constantly adding active and genetic substance into the medium of the tissue culture is helpful to the normal plantlet growth.
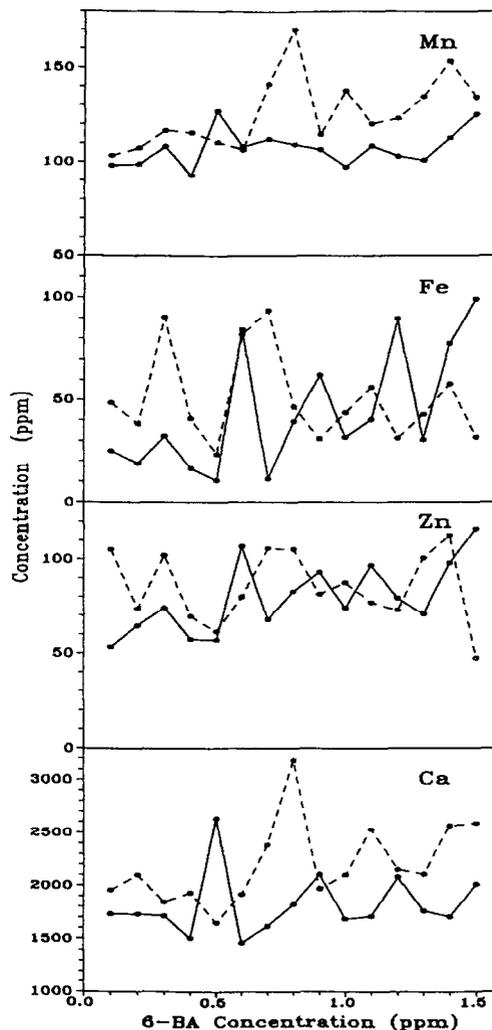
Fig. 4. Variation of the elemental concentration of carnation tissues with 6-BA concentration, showing the absorption of Mn, Fe, Zn and Ca for both the normal (solid line) and vitrified carnations (dashed line). Points shown are the data obtained from the PIXE analysis.

4. Conclusion

The vitrification phenomenon in plant cultures has been observed for years. Despite of efforts of many investigators, a complete understanding of the malformation problem is still lacking. In the present work, we have obtained evidence that the vitrification process is related to the amounts of trace elements of the plant tissue in vitro culture. With the use of modern PIXE technique, we determined precisely the trace element contents. Our results are believed to provide useful information to explore the malformation problem. We summarize our findings as follows:

(1) The vitrification process leads to a significant

change of the trace element concentrations of K, Ca, Fe and Zn. Their concentrations are considerably higher in the vitrified carnations as compared to the analysis of normal ones.

(2) A culture medium maintained at a low concentration of auxin and/or low temperatures is favourable to the growth rate of normal carnation.

(3) The extracted juice of normal carnation has a curing effect on vitrification. Its effect on vitrification is similar to that due to an active and genetic substance. Further investigations on this point are clearly needed.

Acknowledgement

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