Studies on the Varietal Differentiation of Frost Resistance in the Tea Plant

II. Artificial hardening and dehardening experiment with frost resistance

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Synopsis. Using five varieties of the tea plant including a triploid, artificial hardening and dehardening treatments were made. In both treatments the change of osmotic pressure, moisture, and several chemical constituents such as sugars, tannins and nitrogens in leaves was observed to accompany the change of frost resistance. These results revealed that osmotic pressure in leaf cells and the content of moisture, total sugars and water soluble protein had a very intimate relation with frost resistance.

Introduction

The authors reported in the previous paper on the seasonal change of frost resistance in the tea plant and related change of osmotic concentration, water, sugars, tannins, and nitrogens with special reference to the varietal differentiation (SIMURA and SUGIYAMA 1965). On the other hand there are several methods for controlling frost injury by artificial treatment. The artificial hardening (LEVITT 1957; SIMINOVITCH and BRIGGS 1953) and dehardening are usually done. Chemicals are also used for the purpose of increasing frost resistance. Thiouracil is known to increase protein nitrogen in olive (KESSLER 1956) and seems to increase slightly cold resistance in alfalfa (JUNG 1962), although the effect of sucrose (LEVITT 1959) and dimethylaminomaleamic acid (PARKER 1963) on frost resistance are actually not very clear.

The present work was done in several experiments with artificial hardening and dehardening of the tea plant in relation to frost resistance, osmotic concentration and chemical constituents in leaves with special reference to the varietal differentiation.

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Materials and Methods

The experiments for artificial hardening were done from October 1962 to January 1963, and the experiments for artificial dehardening were done from January to April 1963.

Four varieties were used; Yabukita and Y-3 belong to Japanese origin and Beniho-mare and X-10 are hybrids between Assam and China. All of them are 6 or 7 years old. Fully grown leaves were collected from 5 bushes in each variety, and used for the following investigations.

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(1) Artificial hardening. Branches with several leaves were cut off and the cut ends were completely packed with polyethylene paper to prevent them from desiccation. They were kept at 0 °C for 7 days in the freezer.

(2) Artificial dehardening. Branches with leaves cut off by the same manner in the case of hardening were cultured with water at 20 °C for 7 days in the phytotron.

(3) Frost resistance. As the authors' previous work leaves of each variety were placed into the freezer kept at 0 °C for 3-6 hours and then kept at -10 °C or -20 °C for 1-2 hours. After freezing the leaves were cultured with water for 1-2 days at room temperature (20-25 °C). Frost injury was indicated as the grade from 0 to 10 by estimating the area of dead surface over the leaves which was in proportion to frost injury.

(4) Total sugars and reducing sugars. They were determined spectrophotometrically by SOMOGYI's method.

(5) Tannins. By the usual process they were spectrophotometrically determined.

(6) Nitrogens. They were determined by the usual Kjeldahl method. Soluble nitrogens and water soluble protein were determined by the method of SIMINOVITCH and BRIGGS (1953).

Experimental Results

(1) The effect of hardening and dehardening on frost resistance.

Almost no effect of artificial hardening on frost resistance was observed before September. In the beginning of October, however, the effect began to appear and as shown in Table 1, when five varieties were treated with hardening, the effect was manifest at three varieties except X-10 and U-22. X-10 is an Assam hybrid and the weakest in frost resistance. On the contrary U-22 is triploid and the strongest variety. It got higher frost hardness under the natural condition already in early October than other varieties, and although the effect of artificial hardening was not manifest at this

Table 1. The effect of hardening on frost injury

Date	Variety	Yabukita		U-22		Y-3		Benihomare		X-10	
		N	H	N	H	N	H	N	N	N	H
1962	Oct. 1	4	3	2	3	4	3	6	4	6	6
	6	4	3	2	2	4	3	6	5	5	5
	16	4	3	2	1	4	3	5	4	5	5
	25	3	2	2	1	3	2	4	3	5	4
	Nov. 12	2	1	—	—	3	3	3	2	4	3
	21	2	1	—	—	3	2	3	2	4	3
	Dec. 13	2	1	—	—	3	2	3	3	4	3
	20	1	1	—	—	3	2	2	1	4	3
1963	Jan. 8	0	0	—	—	2	1	1	1	4	3

U-22 is a triploid variety and others are all diploid. Leaves were kept at 0 °C for 3 hours, then at -10 °C for an hour and finally at room temperature for 2-3 days. Frost injury was graded from 0 to 10 (0: no injury; 1: slight injury; 10: full injury and so on). N: natural series; H: hardening series.

time, towards the middle of October, when hardened, it showed the highest frost hardness. To the authors' regret they could not continue the experiment with this variety after November.

After the end of October all the varieties got hardened when they were artificially treated with hardening. From the end of December to January all the varieties became naturally hardened, and almost no difference of frost injury was found between the natural

Table 2. The effect of dehardening on frost injury

Date	Variety		Yabukita		Y-3		Benihomare		X-10	
	N	D	N	D	N	D	N	D	N	D
1962 Feb. 15	0	2	1	3	1	2	2	3		
28	0	1	1	2	1	3	2	3		
March 3	0	1	0	2	2	3	2	3		
11	0	2	0	1	2	3	2	3		
18	0	2	1	3	1	2	2	3		
23	0	2	1	2	1	2	2	3		
April 19	2	2	2	3	2	3	3	3		
Dec. 21	1	2	3	4	3	3	4	4		
1963 Jan. 8	0	2	2	3	1	2	4	4		

Leaves were kept at 0°C for 3 hours, then at -10°C for an hour and finally at room temperature for 2-3 hours. Frost injury was graded from 0 to 10. N : natural series. D : dehardening series.

and hardening series. Then in natural conditions Yabukita showed the strongest of four varieties tested ; Y-3 and Benihomare intermediate and X-10 the weakest.

Now all the varieties got fully hardened from January to February in the natural condition and at this period a lot of variation in frost resistance was found among varieties according to their inherent characters. Thus at this period the artificial dehardening treatment gave remarkable effect upon frost resistance, and all the varieties decreased frost hardness as shown in Table 2.

(2) The effect of hardening on osmotic concentration.

As shown in Table 3, at two varieties-Yabukita and Benihomare no effect of artificial hardening was observed in August. In September, however, the slight effect began to appear, and from October to November, as frost resistance became higher, osmotic concentration was manifestly enhanced by hardening treatment.

(3) The effect of hardening and dehardening on water content in leaves.

In October water content in leaves decreased slightly by hardening treatment in all the varieties tested (Table 4), but towards November and December, as the air temperature became lower and the plant became more frost-hardier, the difference of water content in leaves between the natural and hardening series became larger.

As for dehardening, from December to April when the water content of leaves was low, it increased by dehardening treatment as shown in Table 5.

(4) The effect of hardening and dehardening on total sugars and reducing sugars contained in leaves.

Before September total sugars contained in leaves of five varieties did not change by hardening, but they increased by the same treatment in October (Table 6). This hardening effect continued until January, but afterwards no effect was found. Among varieties, Yabukita showed the most content of total sugars until December, but in

Table 3. The effect of hardening on osmotic concentration in leaves

Date	Variety		Yabukita		Benihomare	
	N	H	N	H	N	H
1962 Aug. 24	0.35M	0.35M	0.35M	0.35M		
Sept. 2	0.4	0.4	0.35	0.4		
12	0.4	0.4	0.35	0.4		
26	0.35	0.4	0.35	0.4		
Oct. 12	0.4	0.45	0.3	0.4		
25	0.35	0.45	0.35	0.4		
Nov. 11	0.5	0.55	0.4	0.45		
32	0.5	0.6	0.45	0.5		
30	0.5	0.6	0.5	0.55		
Dec. 6	0.5	0.65	0.45	0.6		
1963 Jan. 16	0.7	0.75	0.6	0.7		
Feb. 10	0.7	—	0.6	—		
March 17	0.55	—	0.5	—		
April 20	0.45	—	0.4	—		

Osmotic concentration is determined by the plasmolysis method. M : Mol concentration. N : natural series. H : hardening series.

Table 4. The effect of hardening on water content in leaves

Variety Date	Yabukita		Y-3		Benihomare		X-10	
	N	H	N	H	N	H	N	H
1962 Oct. 1	61.8 %	61.7 %	61.9 %	61.0 %	61.6 %	61.1 %	61.9 %	61.5 %
6	61.7	61.0	61.6	61.2	61.6	61.3	62.0	61.5
16	61.7	60.9	61.7	60.6	61.7	61.0	61.9	61.3
25	61.2	59.4	61.5	60.7	59.7	60.5	61.9	61.1
Nov. 12	59.0	57.1	60.5	58.9	59.8	57.9	61.3	60.6
21	59.1	56.9	60.2	59.1	59.8	58.0	60.9	59.9
Dec. 13	58.9	56.9	59.8	58.1	59.7	58.1	60.5	58.9
20	59.0	57.1	59.4	58.6	59.3	58.3	59.7	58.8
1963 Jan. 8	58.8	57.0	59.1	58.2	59.1	58.0	59.4	58.9

N : natural series. H : hardening series.

Table 5. The effect of dehardening on water content in leaves

Variety Date	Yabukita		Y-3		Benihomare		X-10	
	N	D	N	D	N	D	N	N
1962 Feb. 15	57.9 %	59.0 %	58.5 %	59.7 %	58.6 %	59.8 %	58.7 %	60.3 %
28	58.1	59.0	58.8	59.8	58.4	59.7	59.0	60.1
March 3	58.3	59.1	58.6	59.7	58.7	59.9	59.1	60.5
11	58.1	59.4	58.5	59.8	58.8	60.1	58.9	60.1
18	57.9	60.3	58.2	59.9	58.6	60.2	59.1	60.4
23	58.1	59.9	58.5	60.1	58.8	60.0	59.0	60.3
April 9	58.5	59.8	58.6	60.1	58.7	59.9	59.3	60.0
Dec. 21	59.0	57.2	58.9	60.0	59.6	58.9	59.4	61.8
1963 Jan. 8	58.8	59.8	59.0	61.6	59.1	61.5	59.4	61.7

N : natural series. D : dehardening series.

Table 6. The effect of hardening on total sugar content in leaves

Variety Date	Yabukita		U-22		Y-3		Benihomare		X-10	
	N	H	N	H	N	H	N	H	N	H
1962 Oct. 1	67.3	70.1	65.2	68.8	62.0	63.4	66.0	65.9	60.9	62.8
6	67.8	70.1	67.8	70.1	62.8	64.1	65.7	67.0	61.9	63.1
16	68.9	77.4	68.0	69.9	64.3	66.0	65.9	68.4	63.0	65.8
25	69.7	79.4	70.5	79.8	64.0	67.8	65.4	67.0	65.1	69.3
Nor. 13	76.1	85.4	—	—	65.8	69.9	68.7	70.9	65.0	68.1
21	86.1	99.8	—	—	67.8	69.1	68.9	75.0	64.9	67.3
Dec. 13	93.6	99.8	—	—	66.9	69.8	70.8	77.6	65.7	68.9
20	92.3	99.9	—	—	75.6	80.9	81.6	88.7	71.6	75.4
1963 Jan. 8	97.7	99.7	—	—	90.1	98.7	93.0	99.8	80.9	87.6

Figures indicate mg/g dry weight of leaves. N : natural series. H : hardening series.

January, when hardened, almost no difference of the content of total sugars could be found at each variety except X-10, although under natural condition Yabukita showed the most total sugars.

Table 7. The effect of dehardening on total sugars content in leaves

Date	Variety	Yabukita		Y-3		Benihomare		X-10	
		N	D	N	D	N	D	N	D
1962 Feb.	15	106.0	99.8	101.5	98.6	102.6	99.7	98.1	96.7
	28	102.1	99.7	98.9	99.0	99.7	98.1	99.5	97.4
March	3	100.7	98.5	99.2	97.4	98.9	96.5	98.7	95.4
	11	99.0	97.1	97.8	96.1	97.8	99.0	97.1	99.6
	18	98.7	96.0	95.8	95.7	98.0	95.4	97.1	95.3
	23	99.7	95.8	94.9	92.3	96.7	94.6	98.0	94.2
April	19	72.3	71.1	76.1	75.3	72.8	71.9	70.1	71.4
Dec.	21	97.6	98.6	80.9	81.3	76.8	74.9	72.8	70.9
1963 Jan.	8	99.8	95.0	93.8	92.1	91.1	90.5	81.5	82.1

Figures indicate mg/g dry weight of leaves. N : natural series. D : dehardening series.

Table 8. The effect of hardening on reducing sugars content in leaves

Date	Variety	Yabukita		Y-3		Benihomare		X-10	
		N	H	N	H	N	H	N	H
1962 Oct.	1	6.80	7.00	6.65	6.70	6.49	6.77	6.15	6.11
	6	6.91	6.99	6.59	6.81	6.52	6.87	6.21	6.41
	16	7.18	7.22	6.79	6.90	6.65	6.74	6.38	6.57
	25	7.23	7.40	6.88	6.91	6.72	6.89	6.50	6.77
Nov.	12	7.78	7.98	7.01	7.39	7.80	7.98	6.77	6.98
	20	8.18	8.56	7.89	7.98	8.34	8.67	6.85	7.01
Dec.	13	8.50	8.97	8.17	8.50	8.81	9.01	7.78	7.98
	20	8.81	8.95	8.56	8.95	8.87	9.12	7.98	8.05
1963 Jan.	8	8.76	9.34	8.51	8.90	8.91	9.25	8.15	8.47

Figures indicate mg/g dry weight of leaves. N : natural series. H : hardening series.

Table 9. The effect of dehardening on reducing sugars content in leaves

Date	Variety	Yabukita		Y-3		Benihomare		X-10	
		N	D	N	D	N	D	N	D
1962 Feb.	15	8.79	8.71	8.65	8.45	9.01	8.79	8.12	8.05
	28	8.81	8.67	8.57	8.40	8.97	8.80	7.89	8.10
March	3	8.95	8.70	8.41	8.34	8.76	8.54	8.09	7.89
	11	8.67	8.55	8.50	8.43	8.61	8.46	7.98	7.67
	18	8.70	8.79	8.42	8.30	8.67	8.51	7.86	7.73
	23	8.67	8.55	8.36	8.11	8.34	8.19	7.98	7.56
April	19	6.31	6.44	6.11	6.50	6.19	6.30	6.22	6.18
Dec.	21	8.87	8.95	8.65	8.77	8.79	8.57	7.81	7.62
1963 Jan.	8	8.79	8.64	8.49	8.30	8.91	8.60	8.01	7.93

Figures indicate mg/g dry weight of leaves. N : natural series. D : dehardening series.

Reducing sugars in leaves also did not increase by hardening before September, but from October it began to increase more or less in every variety (Table 8). From November to December Benihomare showed more content than Yabukita and other

varieties both in natural and hardening series. From the end of January to March almost no effect of hardening was found upon the content of reducing sugars as well as total sugars in all the varieties tested, while at this period both of them decreased slightly by dehardening treatment as shown in Table 7 and 9.

(5) The effect of hardening and dehardening on tannins content in leaves.

Before September tannins content was not changed by hardening in all the varieties, but from October on, it decreased by hardening.

The effect of dehardening upon tannins content was, however, not definite. At Yabukita the content was always increased by dehardening, while at Benihomare it decreased after March and at Y-3 and X-10 it was variable.

(6) The effect of hardening and dehardening on nitrogens content in leaves.

Total nitrogen in leaves was not changed in any season by hardening. Water soluble nitrogen also was not changed before September, but after October it was slightly decreased. On the other hand water soluble protein increased in any variety from October to January by hardening (Table 10). In the case of water soluble protein, Yabukita and U-22 which were higher in frost resistance showed larger content than other varieties.

As for dehardening, from December to January dehardening did not show so manifest effect upon the content of three kinds of nitrogens in four varieties, although as shown in Table 11, water soluble protein were inclined to decrease by dehardening in most varieties tested.

Table 10. The effect of hardening on water soluble protein content in leaves

Variety		Yabukita		U-22		Y-3		Benihomare		X-10		
		N	H	N	H	N	H	N	H	N	H	
1962	Oct.	1	1.92	2.04	1.88	1.95	1.68	1.75	1.53	1.67	1.77	1.76
		6	1.99	2.16	2.05	2.23	1.77	1.69	1.65	1.84	1.71	1.87
		16	2.13	2.21	2.19	2.37	1.98	1.80	1.66	1.78	1.66	1.62
		23	2.38	2.41	2.17	2.29	1.79	1.96	2.00	1.98	1.77	1.85
	Nov.	12	2.58	2.87	—	—	2.10	1.94	2.01	2.19	1.95	2.03
		21	2.43	2.61	—	—	2.03	2.14	1.98	1.87	1.98	2.05
	Dec.	13	2.46	2.49	—	—	1.98	2.05	1.95	2.08	1.87	2.10
		20	2.51	2.53	—	—	1.88	1.98	1.98	2.10	1.89	2.07
1963	Jan.	8	2.13	2.25	—	—	1.83	1.99	1.89	1.97	1.85	2.07

Figures indicate mgN/g dry weight of leaves. N: natural series. H: hardening series.

Table 11. The effect of dehardening on water soluble protein content in leaves

Variety Date		Yabukita		Y-3		Benihomare		X-10	
		N	D	N	D	N	D	N	D
1962	Dec. 13	2.46	2.25	1.95	1.89	1.93	2.00	1.85	1.94
	20	2.56	2.48	1.92	1.98	1.88	1.90	1.87	1.81
1963	Jan. 8	2.13	2.15	1.86	1.80	1.83	1.87	1.85	1.82

Figures indicate mgN/g dry weight of leaves. N: natural series. D: dehardening series.

Discussion

The authors' previous paper (1965) revealed that the tea plant began to increase frost resistance from the end of September, accompanied by the decrease of water content in leaves and the increase of osmotic concentration in the leaf cells, and simultaneously as the plant became frost-hardier, total sugars and water soluble protein contained in leaves increased, while the content of tannins, total nitrogen and water soluble nitrogen decreased.

This relation was also demonstrated in the present study by the artificial hardening and dehardening treatment. SAKAI (1961) stated that in early September (at Hokkaido), when the water content in the cortical cells of mulberry twig decreased, the artificial hardening became effective. In the tea plant, however, it is after October (at Tokai district, middle part of Japan), when the water content in leaves decreased and the artificial hardening became manifestly effective, and from this time frost resistance increased further by hardening, accompanied by the increase of osmotic concentration in the leaf cells and the content of total sugars, reducing sugars and water soluble protein in leaves. On the other hand, by hardening, water, tannins, total nitrogen and water soluble nitrogen contained in leaves decreased further slightly. In the meantime from December to February when the plant was fully hardened under natural conditions, artificial dehardening became effective, and frost resistance decreased by dehardening at all the varieties tested, accompanied by the slight increase of water content and the slight decrease of total sugars and water soluble protein in leaves, while the content of reducing sugars, tannins, total nitrogen and water soluble nitrogen showed no definite relation by dehardening.

From this result and the authors' previous paper (1965) it is likely that the water content in leaves, osmotic concentration in the leaf cells and the content of total sugars and water soluble protein in leaves have intimate relation with frost resistance in the tea plant, although the content of tannins, total nitrogen and water soluble nitrogen could not be found to have any relation with frost hardness.

With regards to total sugars in relation to frost resistance, the present results agree with those of LEVITT (1957, in cabbage, saxifraga, wheat and pines), SIMINOVITCH and BRIGGS (1953a in the bark of black locust tree), PARKER (1959, 1959/1960, in conifers and white pines). All of them recognized that the increase of total sugars in the cells is essential for the increase of frost hardness. Concerning the role of sugars in frost resistance, LEVITT (1957, 1959), SCARTH and LEVITT (1958) and HEBER (1958, 1959) stated that sugars stored in the cell sap decreased the water content to freeze at a given temperature by lowering the freezing point and served to maintain a level of hydration necessary for living of plant cells. Further HEBER and ULLRICH (1958) pointed out the existence of a correlation between the content of total sugars in chloroplasts and the increase of frost resistance in wheat, and described that chloroplasts were inhibited in vitro from freezing by adding sucrose.

Regarding water soluble protein, SIMINOVITCH and BRIGGS (1953 b) investigated the behavior of water soluble protein and sugars in the twig bark of black locust, and

made clear that the increase in frost resistance was closely proportional to the increasing amount of water soluble protein, but not to that of sugar content. SAKAI (1962), however, opposed to their elucidation, and from his studies on the twig bark of mulberry tree, he stated that, whenever the artificial frost-hardening in the cortical cells is effective, the increase in frost-hardiness is closely proportional to that in their sugar content, but the increase in frost-hardiness is not accompanied by any increase in their soluble protein, provided that they are artificially chilled. Further from the fact that from late September to late October, the amount of soluble protein remarkably increases, he pointed out that the accumulation of soluble protein in cells seems to be prerequisite to accomplishing the natural frost-hardening in woody plants. HEBER (1959) also stated that in the chloroplasts of wheat leaves sugars and water soluble protein are two main factors to determine frost resistance.

Now in the present study, from the fact that total sugars and water soluble protein simultaneously increased by hardening, and by dehardening total sugars clearly decreased, although water soluble protein did not so manifestly decreased. It can be possibly said that both total sugars and water soluble protein play an important role in frost resistance in the tea plant.

Summary

Five varieties of the tea plant were used for the experiments with artificial hardening and dehardening in relation to frost resistance and its related change of osmotic concentration in the leaf cells and the constituents of leaves such as tannins, sugars, and nitrogens.

It was after October when the artificial hardening became effective, and whenever the artificial hardening was effective, frost resistance was increased by hardening, accompanied by the increase of total sugars and water soluble protein in the leaf cells. Thus the content of total sugars and water soluble protein have the intimate relation with frost resistance. From December to February when the plant became fully frost-hardy, the artificial dehardening was effective, and frost resistance decreased by dehardening treatment. Then accompanied by the decrease of frost resistance total sugars clearly decreased, although water soluble protein did not so manifestly decrease.

On the other hand, tannins, total nitrogen and water soluble nitrogen contained in leaves showed no definite relation with frost resistance.

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チャの耐凍性の品種間差異に関する研究

II. 人工低温処理が耐凍性に及ぼす影響

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チャの葉を人工的に低温および高温処理したとき、耐凍性と葉の細胞の浸透圧、水分その他の化学成分が受ける影響を調べた。供試材料は日本種として、ヤブキタ、Y-3、U-22 の 3 品種、インド雑種として、X-10、ベニホマレの 2 品種を用いた。このうち U-22 は 3 倍体 ($2n=45$) で、他はいずれも 2 倍体 ($2n=30$) である。人工低温処理は 0°C で 7 日間、高温処理は 20°C で 7 日間材料を保存した。

10 月から 12 月中旬までに低温処理を行うと、すべての品種で耐凍性が増大したが、それ以後は増さなかった。自然状態で耐凍性の大きい 1 月から 3 月にかけて、高温処理を行なうと、すべての品種で耐凍性が減少した。耐凍性が増大する 10 月から 12 月にかけて低温処理を行うと、葉の細胞の浸透濃度は増加し、葉の水分含量はわずかながら減少した。1 月および 2 月の葉の浸透濃度高く、水

分が少ない時期には、高温処理により、浸透濃度は減り、水分は増した。

低温処理により耐凍性が増大するとき、すべての品種の葉の全糖含量が増加したが、還元糖の含量と低温処理の効果は明かでなかった。1~2 月高温処理で、全糖と還元糖の含量はわずかに減少した。葉のタンニン含量は低温処理で、減少したが、高温処理の影響は品種によって一様でなかった。全窒素含量は低温処理で影響されなかったが、水溶性窒素含量は減少する傾向があつた。水溶性蛋白質はすべての品種で、低温処理により増加した。しかし高温処理の影響は全窒素、水溶性窒素および水溶性蛋白質ともに明かでなかった。

以上の実験から葉の水分、浸透濃度、全糖、水溶性蛋白質等は耐凍性と密接な関係があることが推定できる。