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Fractionation of Iron in Rice Leaf Tissue

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벼 잎 의 철 분 별 정 량

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요 약

0.02M EDTA-2Na와 $Na_2S_2O_4$에 의한 벼잎의 철을 분별정량하는 새방법을 적고이병엽을 사용검정하였다.

1) 시도한 방법은 환원철(Fe^{++}), 산화철(Fe^{+++}), 침전철(PFe) 및 결합철(BFe)로 분별정량할 수 있고 식물조직의 생리적상태를 잘 나타내었다.

2) 생리적으로 가장 알맞은 철풀의 패턴은 $Fe^{+++}>PFe>BFe>Fe^{++}$이고 보통인 때 $PFe>Fe^{+++}>BFe>Fe^{++}$이고 부적합한 때 $BFe>Fe^{+++}>PFe>Fe^{++}$이며 독작용이 있을 경우 $BFe>PFe>Fe^{+++}>Fe^{++}$임이 추정된다.

3) 전철에 대한 각철의 백분율은 환원철이 10이하 산화철과 침전철이 20~40이며 결합철은 20~50이었다.

4) 잎의 상부가 하반부에 비해 불건전한 증상임에도 언제나 환원철이 많은 것은 상반부에 환원철이 크게 관여하는 활동적 대사계가 있는 것을 의미한다.

The chlorotic pear leaves may contain as much or more iron than green leaves of the same age (6). This fact indicates clearly that plant leaves have various forms of iron having different functions. The participation firstly in porphyrin metabolism such as the formation of chlorophyll and enzymes(catalase and peroxidase), secondly in the oxidation reduction system in which nitrate reductase or cytochromes are involved(7), thirdly probably in the structure of subcellular organs(4) or in the conformation of macromolecules(2) may be considered as the role of iron in the physiologically essential forms. The resently purified phytoferritin, a protein-iron complex(9) may be a physiologically favorable form. The functions of iron in plant leaves are not always favorable for plant. Thus bronzing and Akagare of the rice plant has been considered to be due to iron toxicity(11)(13). The iron phosphate precipitated in the veins of leaf(8) is one of the physiologically harmful or toxic form.

For the extraction of active iron in chlorophyll formation 1N HCl was proposed after testing water and 0.5N HCl(6) and it was applied in the rice plant(12). Shim and Vose(10) used 0.1N EDTA-2Na and 0.1N HCl for the investigation of initial products of iron absorbed by rice root. Fractiona tion of iron in tomato leaf tissue with water, methanol, ethyl-acetate, carbon-tetrachloride and two aqueous solutions of chelating agents possessing increasing affinities for iron(3) seems no more succesful than that with 1N HCl, though the results strongly indicated that extractable iron exists in various forms.

The easy and rapid method of iron fractionation as possible as it is in plant through which

we can get some informations on the physiological status of iron pools is necessary for the more precise diagnosis of iron nutrition.

This study was carried out in 1967 for the establishment of new iron fractionation method especially in Akagare rice leaves.

MATERIALS AND METHODS

Plant sampling: Rice leaf blades(*Oryza sativa L, var:* Jinhung) were collected from the field of Crop Experiment Station in Suwon and from a farmer's field in Kuwoon-ri at booting stage. The blades were separated into two groups of the diseased and of the healthy. The diseased leaves from Crop Experiment Station showed moderate reddish yellowing discoloration and tiny brown spots and thus it was strongly suspected as akagare but the healthy leaves had no such symptoms. The leaves from Kuwoon-ri showed only slight yellowing in upper half of leaf blade in the diseased and yellow tint in the healthy and it was suspected as early senescence by unknown factor. Since the healthy leaves were collected at the same time it may contain more younger leaves. By cutting the middle of each leaf blade samples were divided into four groups; diseased upper (DU), diseased lower (DL), healthy upper (HU) and healthy lower portion(HL). Each sample was cut into small picies by sissors and directly used for iron fractionation.

Redox potential (Eh) and pH: Fresh samples were homogenized in Waring Blender with deionized water(1:5w/v) for 5 minutes. Redox potential and pH were measured in homogenate with platinum and glass electrode with caromel electrode for reference, respectively.

Iron fractionation: The fractionation method used in this study as shown in flow diagram(Figure 1) was modified from that for iron in soil(1). Ten grams of fresh leaf blade tissue was used for each analysis. Each sample had three replicates for analysis. One seventh molar solution of EDTA (ethylenediaminetetracetate-2Na) was used to make final concentration to 0.02M with deionized water. Sample was homogenized(sample: extractant=1:15w/v) with Waring Blender for 5 minutes and heated on 70C water bath for 30 minutes and centrifuged at 5000g for 10 minutes. The residue was washed with suitableamount of extractant by centrifugation and supernatants were pooled. Total and ferrous iron (Fe^{++}) in the supernatant was determined by o-phenanthroline with and without hydroxylamine hydrochloride, respectively. Ferric iron(Fe^{+++}) was calculated by subtracting Fe^{++} from total iron. The residue was heated again on 70C water bath for 30 minutes with 2g of $Na_2S_2O_4$, centrifuged, washed as in first extraction, and the superna-

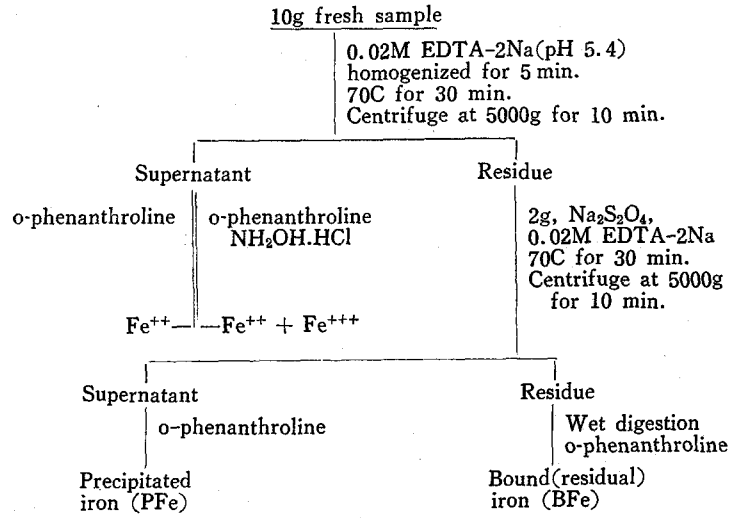

Figure 1. Flow chart of iron fractionation in rice leaf tissue.

tants were pooled. The iron in supernatant was determined as above and designated as precipitated iron (PFe). The residue was wet oxidized with H_2SO_4 and H_2O_2 and iron in this fraction was designated as bound iron(BFe).

RESULTS AND DISCUSSION

Table 1 indicates the iron content of each fraction, it's percent error and percentage to total iron. The tiny brown spot and reddish yellowing symptoms in the diseased leaves may probably be due to iron toxicity since total iron contents on dry weight base in the diseased leaves were over 300 ppm which was reported to exibit the similar iron toxicity symptom(11).

All the percent error of each fraction was less than 10 except one. Statistical analysis of iron fractionation as split-split plot design showed highly significance (at $P=0.01$) in all plot and their interactions. This indicates that iron pool sizes were greatly affected by leaf status and leaf portion. In this analysis main plot, split plot, and split-split plot were healthiness, leaf blade portion and iron forms, respectively. The coefficent of variance of split-split plot was 9.5. This might probably be attributed mainly to the use of iron sissors in preparing samples. The tendency of slightly higher recovery of iron in the strong healthy tissue than the diseased suggests possible contamination of iron from sissors. Statistical analysis indicatse that this extract method is useful for measuring each iron pool size though the precise recovery test is remained.

Shim and Vose(10) reported that 0.1N HCl

Table 1. Iron fractions in diseased and healthy rice leaf tissue.

		Fe^{++}	Fe^{+++}	$\frac{Fe^{++}}{Fe^{+++}}$	PFe	BFe	Total
DU	ppm	1.3	22.5	23.8	37.5	59.3	120.6(355)
	PT	1.1	18.7	19.8	31.1	49.2	100.0
	PE	9.1	3.5	3.3	5.7	3.1	1.5
DL	ppm	0.8	16.5	17.3	23.4	35.7	76.4(329)
	PT	1.1	21.6	22.7	30.6	46.7	100.0
	PE	0	3.8	3.8	12.7	6.2	1.8
HU	ppm	7.1	24.5	31.6	22.1	32.8	86.4(243)
	PT	8.3	28.3	36.6	25.5	37.9	100.0
	PE	4.9	2.1	2.1	6.4	7.2	4.1
HL	ppm	2.3	14.6	16.9	11.9	19.1	47.9(192)
	PT	4.8	30.5	35.3	24.8	39.9	100.0
	PE	0	6.3	6.3	3.4	8.3	5.0

Fe^{++} and Fe^{+++} : 0.02M EDTA extractable
PFe : Precipitated iron extractable with EDTA and $Na_2S_2O_4$
BFe : Bound (residual) iron
DU : Diseased upper half half leaf
HL : Healthy lower half leaf
PT : Percentage to total
PE : Percentage error (standard deviation/mean $\times 100$)
ppm : Fresh weight base
Parenthesis : Dry weight base.

tion while 0.1N EDTA did not. The iron in the first extraction(designated as free iron hereafter) may be mostly free or loosely bound. The percentage of this fraction to total iron seems to be more significant in relation to physiological activity than the absolute amount since we can infer the physiological activity of four rice leaf samples in increasing order of diseased upper leaf(DU) diseased lower leaf(DL) healthy lower leaf(HL) healthy upper leaf(HU), according to the leaf color and position and then this activity order is well agreeable to the order destructed iron com-

plex during extracof the percentages of free iron fraction to total iron. The percentage of free iron fraction which showed around 20 in diseased and about 35 in healthy leaf appears to be close to that of 1N HCl-soluble fraction(12) and also to that of succesive extraction by chelating agents (3). Thus it must contain mostly active iron directly involved in the metabolism.

The determination of ferrous iron in the free iron fraction seems to be doubtful as long as there was no preventive measure of oxidation of iron during analytical process. Taking into account the fact that the healthy leaf showed negative redox potential and had more ferrous iron while the diseased leaf showed positive redox potential(Table 2) and had more ferric iron the ferrous fraction, however, could be considered as a measurable fraction at least useful as relative value for the indication of iron status. The EDTA-extractant may have retarding effect of oxidation of ferrous to ferric iron. The analytical significance of ferrous fraction may be evaluated by measuring ferrous and ferric form with Mössbauer spectroscopy (5). For the determination of iron in the free fraction a greenish tint of supernatant due to decomposed chlorophyll gave slight interfering effect but it was not considered.

Table 2. Redox potential and pH of rice leaf homogenates.

	DU	DL	HU	HL
Eh(mv)	+140	+60	−10	−95
pH	5.75	6.25	6.40	6.40

DU: diseased upper half, HL: healthy lower half

The third fraction extracted under highly reduced condition by using 2g of $Na_2S_2O_4$ may probably be ferric compounds such as ferric phosphate precipitated in the veins (8). Thus it could be designated as precipitated iron(PFe). Only easily reducible oxidized iron compounds might be mostly extracted. Since reducing condition could cause destruction of leaf tissues to some extent the possibility of extraction of bound iron can not be eliminated. This fraction was greater than free ferric fraction in the diseased leaf while it was reverse in the healthy leaf. If this fraction includes mostly the easily reducible ferric compounds this fraction may have some equilibrium with the free ferric iron fraction and thus this equilibrium status between two ferric fractions might have metabolic significance in iron nutrition.

The last fraction(BFe) was residual and it may probably contain strongly bound iron in plant tissues which may have some significant role in structure(4) but also highly oxidized iron compounds to some extent. What extent of the residual iron could be structural was beyond this investigation. If structural iron is thought to be essential, it's content in the diseased tissues must not exceed that in the healthy tissue though there could be variation in different part of leaf. Then the excess iron could be thought as highly oxidized iron compounds.

Table 3. Iron fractions in diseased and healthy rice leaf tissue.

		Fe^{++}	Fe^{+++}	$\frac{Fe^{++}}{Fe^{+++}}$	PFe	BFe	Total
DU	ppm	3.82	14.2	18.0	24.3	13.9	56.1(189)
	PT	6.8	25.3	32.1	43.2	24.7	100.0
DL	ppm	0.71	18.5	19.2	14.3	9.2	42.7(213)
	PT	1.6	43.4	45.0	33.5	21.5	100.0
HU	ppm	3.37	14.3	17.7	15.5	11.2	44.4(124)
	PT	7.6	32.3	39.9	35.0	25.1	100.0
HL	ppm	1.85	14.5	16.3	9.3	8.6	34.2(132)
	PT	5.4	42.3	47.7	27.1	25.2	100.0

DU: diseased upper half, HL: healthy lower half, Fe^{++} and Fe^{+++}: 0.02M EDTA extractable, PFe: extractable with EDTA and $Na_2S_2O_4$, BFe: residual iron, ppm: on fresh weight base and dry weight base in parenthesis, PT: percentage to total.

The proposed method showed clear difference in the four fractions of iron in the diseased and healthy leaf tissues as mentioned above, that is, both the content and the percentage of each fraction was BFe>PFe>Fe^{+++}>Fe^{++} in the diseased leaf and BFe>Fe^{+++}>PFe>Fe^{++} in the healthy leaf. Both ferrous and ferric fraction were tend to increase with the increase of physiological activity while both precipitous and bound iron fraction were tend to decrease(Figure 2).

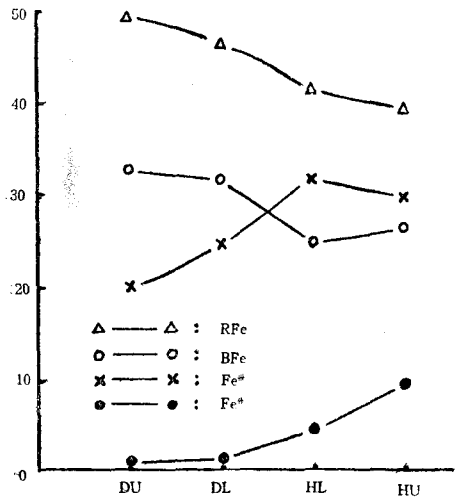

Figure 2. The change of iron pool size with the physiological status of rice leaf.

notice: DU: upper part of diseased leaf,
HL: healthy lower part,
PFe: precipitated iron,
BFe: bound iron.

The results of iron fractionation in the other rice leaf sample taken from farmer's field in Kuwoon-ri was shown in Table 3. The iron contents in each fraction showed well agreement with healthy rice leaf tissue of first sample except that BFe fraction was less than PFe fraction. In this case the difference between two leaf samples was not much different except more yellowing in diseased leaf. The fact that there was little difference between diseased and healthy leaves indicates that the symptoms of leaf was not caused by iron toxicity and thus the iron status in these leaf samples was in better condition than previous healthy leaves considering the lower total iron content, the greater percentage of free iron fraction and less percentage of bound iron fraction in these samples than those in first healthy samples. According to the appearance of sample the lower half leaf is healthier than the upper half leaf in this case. Thus the distribution pattern of iron pools of Fe^{+++}>PFe>BFe>Fe^{++} inlower half of leaf indicate more physiologically favorable one than PFe>Fe^{+++}>BFe>Fe^{++} in the upper half. Consequently we can infer the pattern of PFe>Fe^{+++}>BFe>Fe^{++} is more favorable than the pattern of BFe>Fe^{+++}>PFe>Fe^{++} in healthy leaf of previous sample.

The fact that the percentage of BFe fraction were succesively decreased from 50 to 45 and 40 (Table 1) and to 25 and 20(Table 2) according to physiological status of leaf indicates that the proposed method is effective more in the fractionation of the precipitated or bound form of iron. The effectiveness of this proposed method could be more precisely proved by using the iron-fed plant leaves at various levels and different forms of iron with a recovery test.

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SUMMARY

A new method for the measure of iron pools using 0.02M EDTA and $Na_2S_2O_4$ was tested on Akagare diseased and healthy rice leaf tissue

1) The proposed method could fraction iron into four fractions; ferrous iron(Fe^{++}), ferric iron (Fe^{+++}) precipitated iron(PFe) and bound iron(BFe) well indicating the physiological status of tissue.
2) The pattern of iron pools appears to be Fe^{+++}>PFe>BFe>Fe^{++} in most physiologically favorable status of iron, PFe>Fe^{+++}>BFe>Fe^{++} in favorable status, BFe>Fe^{+++}>PFe>Fe^{++} in unfavorable status and BFe>PFe>Fe^{+++}>Fe^{++} in toxic status.
3) The percentage of each fraction to total iron was less than 10 for Fe^{++}, 20 to 40 for Fe^{+++} and PFe and 20 to 50 for BFe.
4) Ferrous iron was always higher in upper half

leaf, the appearance of which is less healthier than lower half indicating that there is more active metabolic system in which ferrous iron is involved.

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