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Effects of Physiological and Morphological Characteristics of Root Tips Excised from Rice Seminal Roots on Subsequent Growth *in vitro**

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Abstract : Tissue culture of excised root is a useful method with which genetic variation of plant root itself can be determined without the influence of shoot. We aimed to determine the effects of age and elongation rates of the seminal root axis of rice on subsequent growth in a culture medium. Taichung Native 1 (indica type) and Yukara (japonica type) were used in the experiments. The seminal root tips (1-cm-length) were sampled every day after bedding on agar and their morphological and physiological characteristics were monitored by recording the persence of lateral roots and primordia, dry weight and C・N content. Root tips with different ages or elongation rates were then cultured, and subsequent growth was observed after three week culture. Root tips which were older and had lower elongation rates showed inferior root growth in both cultivars. When a more than three-day-old seminal axis on which lateral roots started to emerge was excised, less L-type first order lateral roots was produced than that grown on the two-day-old axis, and this affected the total root number and length. The dry weight or C・N content of excised segments decreased as the excision day, and these characteristics showed a close correlation with the subsequent root growth. Thus, we concluded that the excision of root tip segments at an earlier stage ensures excellent development of seminal root system *in vitro*.
Key words : Age, C・N content, Elongation rate, Excised root culture, Lateral root, *Oryza sativa* L., Rice, Root tip segment.

水稲種子根の根端の採取時における生理的・形態的特性が培養条件下での生育に及ぼす影響：泉　泰弘・河野恭廣・青島孝則・山内　章・飯嶋盛雄（名古屋大学農学部）

要　旨：根端培養法は，地上部の影響の除去が可能な点で，根の生育における品種間の遺伝変異を検出するには優れた実験系である．しかし，伸長速度の異なる種子根から採取したという前歴や生理的エイジの違いが，根系発達に大きな影響を与えている可能性がある．そこで本研究では，供試する根端の生理的・形態的特性が，その後の培養条件下での生育に及ぼす影響を調査した．インド型水稲品種，台中在来1号と日本型水稲品種，ユーカラを用い，播種後2～6日目まで根端（長さ1cm）を毎日採取し，乾物重，炭素・窒素含量の経日変化を追跡した．併せて，別に採取した種子根軸上の側根の出現と側根原基の形成も調査した．さらにそれらの根端を3週間培養した後の根系形態の定量的解析を行った．両品種とも播種後日数が経過した根端，伸長速度の小さい種子根から採取した根端ほど生育に劣る傾向を認めた．また種子根軸上に側根や側根原基が観察された3日目以降に根端を採取した場合，L型1次側根の発生は2日目に採取した場合に比べて極めて少なく，それがとくに総根数・総根長に劣る原因と考えられた．切断時の根端の乾物重，C・N含量は日数経過とともに減少し，その後の根系発達と密接な相関関係を有することが示唆された．これらの結果から，培養に供試する根端をできるだけ早い時期に採取することによって，培養系での良好な種子根系の発達が得られると結論した．
キーワード：イネ，エイジ，根端，根端培養法，伸長速度，水稲，側根，炭素・窒素含量．

Evaluation of crop root system morphology is important since it affects the growth and yield performance through root functions. However, it is generally difficult to characterize the root system morphology of a certain species or cultivar, especially that grown under field conditions. This is mainly because the root system morphology is known to be greatly affected by various environmental factors, which are difficult to control or evaluate[10].

One of the most promising experimental techniques to overcome the problem would be the excised root culture method. Using this method, the environmental factors that would affect root growth can be considerably controlled. In addition, the excised roots can be grown without the influence of the shoot, or

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interaction with other roots[2,11]. The method, however, has been shown to have some problems.

Excised-cultured roots develop in a unique way, i.e., the phenotype is expressed by the genomes, which control only root growth. Hence, the physiological and morphological conditions of the excised root tip would greatly affect subsequent root growth during the culture. However, their influences have not been sufficiently investigated so far.

Kono *et al.*[8] reported that nitrogen and phosphorus supply from the endosperm to the seminal root of paddy rice decreased from the third day after germination when nodal roots started to emerge. They also observed that the metabolic state of the seminal root changed during the same period, i.e., the root tip changed its growth characteristics from being meristematic to elongative. This fact strongly indicates that the physiological status of root tip to be cultured may have great impact on the subsequent growth *in vitro*. Therefore, serious attention should be paid to this aspect to reasonably compare the morphologies of seminal root system (seminal root axis and lateral roots) among several rice cultivars by using the excised root culture method.

Thus, in this study, we aimed to determine the physiological and morphological characteristics of the seminal root tips at different ages and to assess the effects of these characteristics on their subsequent development *in vitro* for two different rice cultivars.

Materials and Methods

Two rice cultivars, cv. Taichung Native 1 (TN-1, indica type) and cv. Yukara (japonica type) were used in this study. We chose them because the preliminary survey showed that these two cultivars were very different in the root growth *in vitro*.

Husked grains were soaked in 70% Et-OH and 5% sodium hypochlorite solution for 30 seconds and 30 minutes, respectively, for surface sterilization. The seeds were then washed with sterilized water three times. Subsequently, they were soaked in sterilized water for two hours and then placed over a well-sterilized germination bed made of 2% agar in Petridish (9 cm in diameter).

A preliminary experiment using hundreds of seeds from the two cultivars was conducted to determine the average length of seminal root axis at 2, 3, 4, 5 and 6 days after seed bedding. Based on the data from the preliminary experiment, two experiments were conducted, in which the time and way of excision of 1-cm-length root tip segments for culture materials are different. Series 1 (focused on root age of seminal root axis) : from 2 to 6 days after bedding, root tips (culture materials) were excised every day from the seminal root axes (mother roots) which attained the length close to the average value in the preliminary experiment (Table 1). In other words, culture materials were sampled from mother roots which showed the average elongation rate at each sampling day. Series 2 (focused on elongation rate of seminal root axis) : in the same number of days, culture materials were excised from mother roots which attained the same length shown in Table 1 in different number of days. Namely, culture materials were sampled in several sets according to mother root length, within each set, the length of mother roots was same but their elongation rates (number of days required to attain the length) were different. This series of experiment was attempted because variation in the elongation rates of seminal root axis among plants was not negligible.

Surgical scissors were used for excision. Some of the excised root tip segments were

Table 1. Changes in average seminal root axis length (mm) of two rice cultivars grown on sterilized agar with days after seed bedding.

Cultivars	Days after seed bedding				
	2	3	4	5	6
TN-1	17±5	43±9	53±8	63±9	70±8
Yukara	15±6*	24±7	40±11	56±15	62±2

* : Data of seeds that did not produce seminal root were excluded for calculation.

Each value is shown in average±standard deviation.

oven-dried at 80°C for 48 hours and then weighed, and their carbon and nitrogen contents were determined with the use of CHN corder (Yanaco MT-5). The rest of the root tip segments were cultured.

We followed the root culture method proposed by Kawata *et al.*[5], Kim *et al.*[7] and Lai & Lee[9]. Two root tip segments were put into a 100 m*l* Erlenmeyer flask containing 15 m*l* of Lai & Lee's R_2 culture mediun[9] sterilized at 120°C with autoclave for 5 minutes. A total of ten roots from five replicate flasks were used for each sampling. Then the flasks were incubated at 28°C in darkness. The R_2 medium contained only nitrate ions as a nitrogen source.

The cultured seminal root systems were sampled at 3 weeks after transfer to the culture medium. The samples were fixed in FAA solution (70% Ethanol : Formalin : Acetic acid = 18 : 1 : 1). The lateral roots were classified into two categories; L-type lateral root, which is long, thick and able to branch high order lateral roots, and S-type lateral root, which is short, slender and has no branching capacity[8]. The number of L-type and S-type lateral roots, the length of each lateral root of different branching orders and the seminal root axis length were determined manually.

The seminal root axes sampled through two timecourse series were observed under a light microscope to count the number of lateral roots and primordia formed on them.

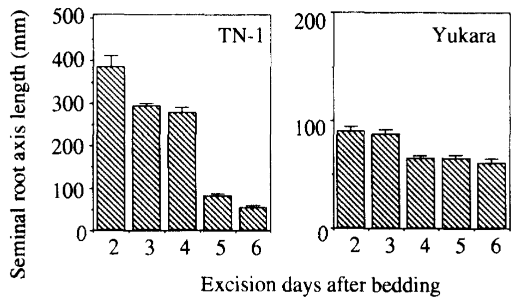

Fig. 1. The seminal root axis length of the 3-week-cultured roots with different ages. Root tips were sampled through the timecourse series 1 (See Materials and Methods). Error bars show standard errors.

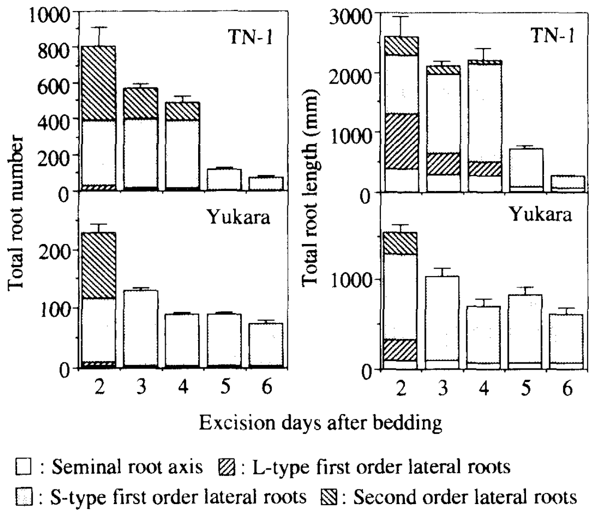

Fig. 2. The total number and length of 3-week-cultured roots with different ages. Root tips were sampled through the timecourse series 1 (See Materials and Methods). Error bars show standard errors of total value.

Results

1. Effect of age

Fig. 1 shows the length of three-week-cultured seminal root axis. In TN-1, the later the excision day was, the smaller the final root axis length was. On the other hand, such a trend was not clearly observed for Yukara. Overall axis elongation was more vigorous in TN-1 than Yukara.

Such trends were also observed for both the number and length of all the root system components in TN-1 (Fig. 2). Comparing the value on day 2 with that on day 4, the total root number was fewer in the latter mainly due to the fewer number of second order lateral roots. This in turn was mainly caused by fewer emergence of L-type first order lateral roots, from which second order lateral roots originated. The differences in the number and length between day 4 and 5 were due to seminal root axis length, and number and length of S-type first order lateral roots. In the case of Yukara, when root tip was excised later than day 2, total root number and length were markedly smaller because only a few L-type first and their concomitant second order lateral roots emerged.

The formation of lateral roots and primordia on seminal root axes started on day 3 for both cultivars and increased in number with the elongation of the seminal root axis (Fig. 3). The increase rate from day 2 to 3 was faster in TN-1 than in Yukara.

Fig. 4 shows dry weight, and C • N content of the culture materials sampled at different days. Dry weight of TN-1 sharply decreased with the delay in time of excision. In accordance with this, C • N content decreased gradually. Yukara also showed these trends, but more slowly than TN-1, as in its mother root length.

2. Effect of elongation rates

The elongation rate of seminal root axis in TN-1 was considerably uniform among the plants sampled each day. Therefore, it was impossible to collect a sufficient number of segments from seminal root axes on different days to evaluate the effect of elongation rates on subsequent growth, so data was not collected. However, in the comparison between groups that showed different elongation rates, the same tendency observed in Yukara were noted in all parameters.

For Yukara, growth of cultured root was very poor when culture materials were sampled from mother root which was longer than 30 mm regardless of excision day. Thus, the comparison was made only for the cultured seminal root systems developed from culture materials which were derived from the shorter

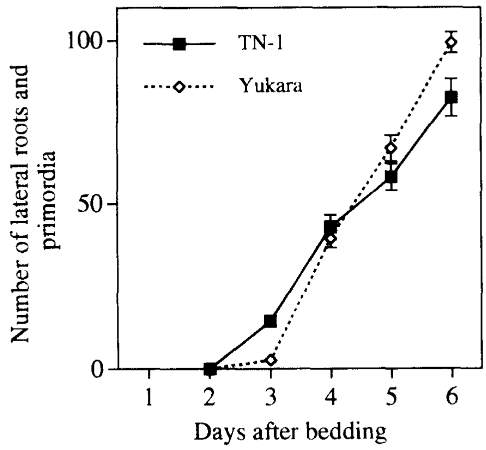

Fig. 3. Changes in sum of the number of lateral roots and primordia recognized on seminal root axis of two rice cultivars sampled through the timecourse series 1 (See Materials and Methods). Error bars show standard errors.

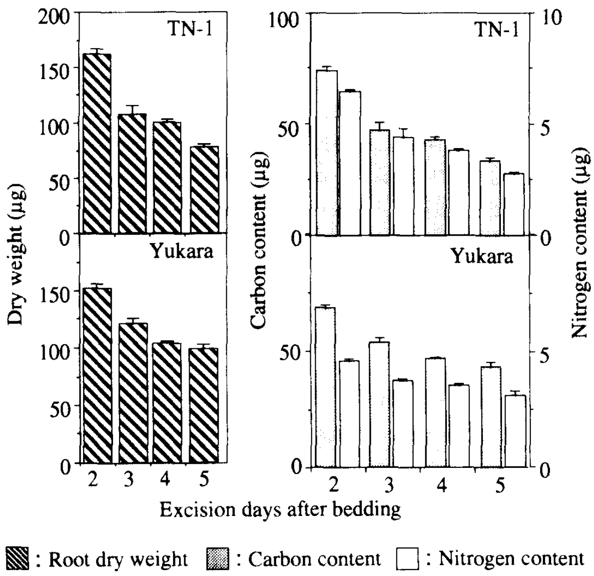

Fig. 4. Differences in dry weight and C • N content per 1-cm root tip segment excised from seminal root axis of two rice cultivars. Root tips were sampled through the timecourse series 1 (See Materials and Methods). Error bars show standard errors.

Table 2. The influence of average elongation rate of seminal root axis till excision on subsequent development in four characters of 3-week-cultured seminal root system of Yukara.

Axis length on excision	Excision day after bedding	Elongation rate (mm/day)	Seminal root axis length (mm)	Number of 1st-L***	Total root number	Total root length (mm)
<20mm*	2	7.5	91±4	7±1	226±18	1541±103
	3	5.4	67±4	1±1	94±11	900±112
	4	4.5	68±4	0±0	99± 8	708± 61
	5	3.6	50±6	not emerged	70± 9	333± 50
20-30mm**	3	8.1	88±4	not emerged	128± 5	1028±105
	4	6.4	83±4	not emerged	123± 5	721± 43
	5	5.9	60±5	not emerged	90± 7	702±122
	6	5.0	45±3	not emerged	52± 3	276± 36

*,** : Root tips were sampled through the timecourse series 2 (See Materials and Methods), i.e., they were sampled from seminal root axes which attained the length close to the average value (Table 1) on day 2 and 3, respectively.

*** : Number of L-type first order lateral roots.

Each value is shown in average±standard error.

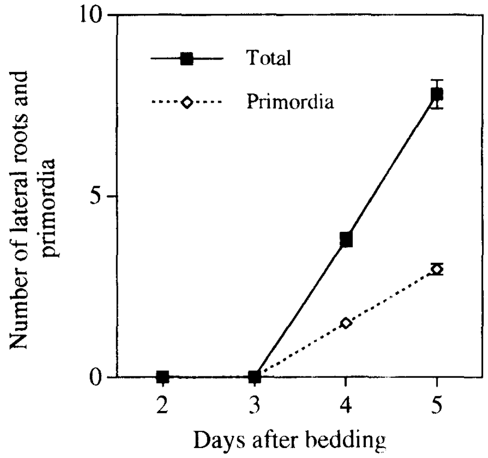

Fig. 5. Changes in the number of lateral roots and primordia recognized on seminal root axis of Yukara sampled through the time-course series 2 (See Materials and Methods). Error bars show standard errors. The average length of seminal root axis was 15.1 mm on day 2, 17.2 mm on day 3, 18.9 mm on day 4, and 17.0 mm on day 5.

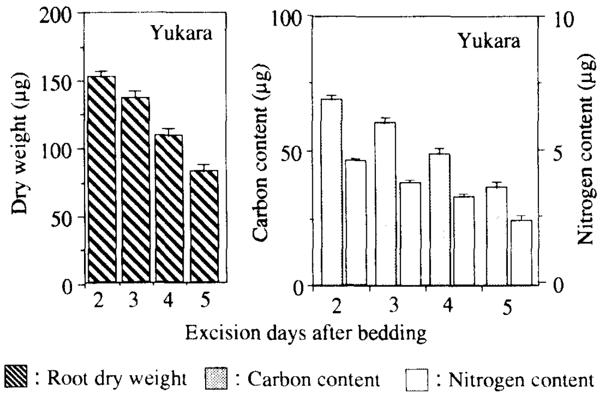

Fig. 6. Differences in dry weight and C • N content per 1-cm root tip segment of Yukara excised from seminal root axes which attained similar length on each excision day. Root tips were sampled through the timecourse series 2 (See Materials and Methods). Error bars show standard errors. The average length of seminal root axis on excision are shown in the note of Fig. 5.

seminal root axes (Table 2). Overall, the cultured root axis length became smaller when the root tip excision delayed. More drastic changes were observed in development of L-type first order lateral roots. When the root tip was excised later than day 2, few L-type first order lateral roots were produced. This difference in turn affected both the total number and length of the root system. Further, no L-type lateral roots were produced on the cultured roots when the mother root was longer than 20 mm. Therefore, the difference in total root number and length were not as drastic as in the former group (less than 20 mm axis length).

The morphological changes of seminal root axis which were distinguished by the presence or absence of lateral roots and primordia (Fig. 5) were observed from day 3 to 4. The results of analysis of 1-cm-length root tip segments of Yukara, whose mother seminal root axis length was almost the same ($<$20 mm) is shown in Fig. 6. It is clear that even though the lengths were similar, the dry weight and C • N content decreased as the time of excision delayed.

Discussion

From the results of the two experiments we found that younger and faster-growing root tips which were greater in dry weight, and richer in C and N contents grew more vigorously *in vitro*.

Kawata *et al.*[6] reported that growth of excised root was improved when culture material was sampled as soon as possible after bedding and sampled from mother root with lower elongation rates. The latter result seems to disagree with ours. Kawata *et al.*, however, focused on the seasonal variation of seminal root axis elongation in relation to seed dormancy. In our case, the target is the individual variation of elongation. Therefore, it appears that there is then no conflict between these two results.

Brown[1] reported that the metabolic activity of excised root tip segment, which is closely related with cell age, dominated subsequent cell division rate in culture system. Our results are firmly supported by this result. From visual observation on the seminal axis, it was also confirmed that the state of the axis changed with time. Although no morphological change was observed in series 2 between day 2 to 3 (Fig. 5), a crucial physiological change might have occurred.

The seminal root system development and C • N content of the culture materials were positively correlated (Table 3). The N content showed closer correlation with root characters than C content. It is noteworthy that N content, which accounts for a lesser portion of dry

Table 3. Correlation between dry weight (DW) and C • N content of 1-cm root tip segment, and two characters of 3-week-cultured seminal root system.

Cultivars	Content	Seminal root axis length	Total root length
TN-1	DW	0.669*	0.524
	C	0.685*	0.546
	N	0.788**	0.669*
Yukara	DW	0.679*	0.896***
	C	0.689*	0.898***
	N	0.713*	0.902***

*,**,*** : 5, 1, 0.1% level of significance.

weight than C, had greater impact on root growth. Therefore, N content seemed to express the physiological state of root tip accurately.

Correlation coefficients between dry weight and C • N content of the culture materials and the cultured root axis length were relatively similar for the two cultivars, but the coefficients for total root length were considerably different. This indicates that the difference between cultivars was mainly in their lateral root development.

Drew *et al.*[3] reported that nitrate concentration in an external medium affected the growth of lateral roots but not the seminal axis of barley in hydroponic culture. R_2 medium contains only nitrate ions as an inorganic nitrogen. Hence, roots need to assimilate inorganic nitrogen for growth except for organic nitrogen available from excised segments themselves. Consequently, it was assumed that the different correlation coefficient in total root length would reflect nitrate metabolic activity. Kim *et al.*[7] suggested that the root of japonica rice cultivars has less nitrogen assimilation capacity than that of indica rice. In this respect, it was suggested that the root growth of Yukara would depend on the organic nitrogen originally contained in the culture material more than TN-1.

In our experiment, very few L-type first order lateral roots were produced on the root tips when excised after day 2, which considerably affected total root growth (Fig. 2 and Table 2). Previously, we reported that the emergence and the development of L-type first order lateral roots required organic nitrogen[4]. On this point, further detailed study of nitrogen metabolism in the excised culture system is needed.

In conclusion, we found that the aging of culture material greatly affects the development of seminal root system *in vitro* in both rice cultivars examined. Hence, it is very important to excise root tip segments from seminal root axes at an early developmental stage as possible. To ensure excellent development of cultured root, a careful seed preparation and a suitable germination method is necessary.

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