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DORMANT BUD FORMATION IN TEMPERATE GRASS SPECIES (POACEAE) CULTURED *IN VITRO* SYSTEM

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Introduction

Grass species of temperate origin are the dominant component of natural pastures and cultivated grasslands, where the grass is the major source of energy supply for animal growth and performance, while the phenological traits in growth and development of grass species produced and conserved in cool and chilly season are of crucial importance.

The dormant bud formation in grass species is known as a pattern of survivals during winter, while it is also one of the basic prerequisites of reestablishment of a photosynthetic canopy after removal of active meristematic tissues[23], for example, by grazing, by cutting, and also by fire, through the activation of axillary buds[11,12]. Therefore, studies on the mechanisms of dormant bud formation in grass plants and of adaptation under environmental stressed conditions for sustaining their lives are of scientific, and agricultural interests[24].

Almost all the works studied previously on grass were concerned with leaf and tiller production, effects of cutting on tillering, regrowth and regenerative capacity[6,8,9,10], and with effects of drought and defoliation on bud viability in field conditions, or in glasshouse[3,4].

However, few data are available for the effects of osmotic stress caused by sucrose, mannitol, and exogenous application of abscisic acid (ABA) on vegetative growth and dormant bud formation. In this report several key grass species of grass family, Poaceae, are selected and practiced in aseptic culture system in order to demonstrate their physiological growth traits, such as development of leaf blade, tillers, and dormant buds.

Materials and Methods

Plant materials

The cultures were carried out on species of perennial forage plants of family Poaceae, such as *Bromus inermis* cv. Baylor, *Bromus inermis* cv. Radison, *Phleum*

pratense cv. Kumpu, *Dactylis glomerata* cv. Okamidori, *Poa pratensis* cv. Baron, and *Festuca arundinacea* cv. Hokuryo.

Aseptic culture and vegetative growth

The seeds for the germination were surface sterilized in 80% (v/v) ethanol for 3 min followed by 1% (v/v) sodium hypochlorite containing a drop of Tween #20 for an hour, and rinsed 3 times in double-distilled sterilized water.

The sterilized seeds were transferred aseptically to sterile plastic Petri dishes (90×20 mm), containing 20 ml the medium, with 20 seeds per dish for germination. Murashige & Skoog's medium (MS)[14], supplemented with 3% (w/v) sucrose, 0.2% (w/v) Gellan Gum was used as a basal growth medium for the germination and the initial vegetative growth. Seeds maintained in the medium were allowed to germinate in dark for a week at 25 °C. Then, for the vegetative growth, three seedlings were transferred aseptically to a culture bottle containing 30 ml of the basal MS medium and were placed in a chamber at 23 ±1 °C under 16 hr photoperiod with a maximum irradiance of 16 W m^{-2} sec^{-1} (equivalent to 0.075 mmol $m^{-2}sec^{-1}$) provided by cool white fluorescent tubes (Sylvania-NEC FL20sw/100v) for 4 weeks.

Plants, cultured *in vitro* for 4 weeks, were clipped at 0.7-1.0 cm upper than root crown, and were allowed to multiply or to form a cluster for 4 weeks in basal MS medium supplemented with 1mg/l BAP (Benzylaminopurine), prior to experimental manipulations (Fig. 1).

Dormant bud induction

After the cultures of 9 weeks, multiplied plants were cut at the 0.7-1.0 cm upper than the root crown, the plantlets were used for the experiments of dormant bud induction as is shown in Fig. 1. The plants were transferred onto MS medium, supplemented with different concentrations of sucrose (0.09M, 0.26M, 0.44M, 0.61M), mannitol (0.09M, 0.26M, 0.44M, 0.61M) and ABA (10^{-5}M, 3×10^{-5}M, 10^{-4}M) and were grown under 16 h photoperiod at 23±1 °C for 6 weeks. The buds formed were transferred to the basal MS medium, and were cultured in 16 h photoperiod at 23 ±1 °C for 4 weeks in order to detect the dormant conditions of those buds.

A metabolic activity and viability of the buds, which did not show any growth, were detected by the TTC (2,3,5-triphenyl tetrazolium chloride) test, as described by Steponkus and Lanphear[18]. One tenth volume of 0.1M succinate, pH 6.0, was added to the incubation mixture for detection of TCA cycle activity. Each experiment was repeated at least twice with four and more replicates.

Growth measurements

The vegetative growth of plants after 6 weeks culture under the experimental medium was measured by determining numbers of tillers, dormant buds, leaves,

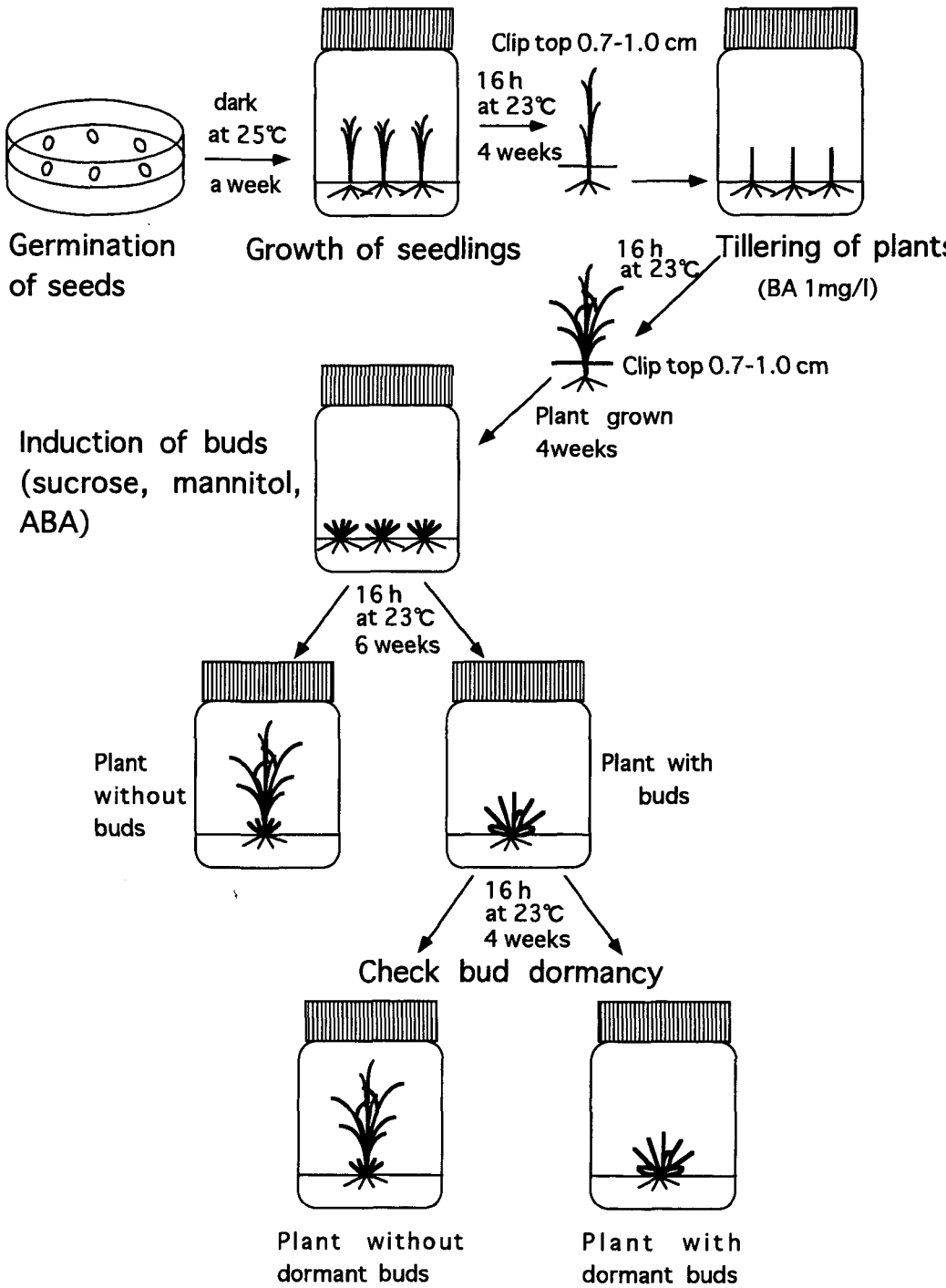

Fig. 1. Scheme of experiment for induction of bud formation in perennial species of grass.

and leaf length, and weights of fresh and dry matters of the excised tillers.

Results

Application of sucrose on growth of grass cultures

The first step of this investigation was to verify the effect of sucrose on vegetative growth and induction of dormant bud formation in selected species of grass, *in vitro*.

The data of the vegetative growth were described by determining fresh and dry matters, leaf initation, leaf length and tillering in 6 separate species of grass plants, cultured in MS medium supplemented with different amount of sucrose. When plants of *Phleum pratense* (Fig. 2, Tab.1), *Bromus inermis* cv. Baylor (Tab. 3), *Bromus inermis* cv. Radison (Tab. 4), *Dactylis glomerata* (Tab. 5), *Poa pratensis* (Tab. 6), and *Festuca arundinacea* (Tab. 7) were aseptically cultured 6 weeks in 16 h photoperiod at 23°C in MS medium supplemented with sucrose, such as 0.09M, 0.26M, 0.44M, and 0.61M (which are equivalent to 3%, 9%, 15%, and 21% sucrose, respectively), the vegetative growth was promoted as the concentration up to 0.26M sucrose, then appeared cessation of growth exhibited by a declination of fresh weight and dry matters per plant, by a reduction of leaf length (Tab. 2) and by a retardation of leaf initation (Fig. 3).

A study of tillering has demonstrated that all of the species examined were remarkably enhanced by sucrose supplementation, although there is a variation

Table 1. Effect of sucrose on growth and bud formation of *Phleum Pratense* cv. Kumpu, cultured 6 weeks in 16h photoperiod at 23°C, *in vitro*

Treatment	Tillers and buds per plant	Dormant buds per plant	Fresh Wt, mg per plant	Dry Wt, mg per plant
0.09M Sucrose	2.11±0.35	0	430.0±40.0	100.0±2.0
0.26M Sucrose	3.54±0.31	0	350.0±22.0	120.0±8.0
0.44M Sucrose	2.89±0.27	1.22±0.12	180.0±18.0	60.0±6.0
0.61M Sucrose	2.12±0.18	0.75±0.09	60.0±2.0	20.0±1.0

Table 2. Effect of sucrose on leaf growth of *Phleum pratense* cv. Kumpu, cultured 6 weeks in 16h photoperiod at 23°C, *in vitro*

Treatment	Leaf number per plant	Leaf length, cm 1-st	2-nd	3-rd	4-rd	5-th	6-th
0.09M Sucrose	4.48	1.72	3.56	5.61	9.19	12.54	14.38
	±0.20	±0.17	±0.30	±0.50	±0.64	±0.77	±8.82
0.26M Sucrose	4.86	1.47	3.03	4.96	5.99	6.15	7.66
	±0.15	±0.15	±0.21	±0.32	±0.39	±0.39	±0.34
0.44M Sucrose	3.17	0.74	1.69	3.16	3.74	7.46	6.91
	±0.18	±0.05	±0.13	±0.23	±0.33	±0.47	±0.54
0.61M Sucrose	2.73	0.68	1.16	0.68	1.16	1.29	0.88
	±0.09	±0.04	±0.07	±0.04	±0.07	±0.06	±0.07

Table 3. Effect of sucrose on growth and bud formation of *Bromus inemis* cv.Baylor, cultured 6 weeks in 16h photoperiod at 23°C, *in vitro*

Treatment	Tillers and buds per plant	Dormant buds per plant	Fresh Wt, mg per plant	Dr Wt. mg per plant
0.09M Sucrose	1.50±0.15	0	470.0±90.0	80.0±10.0
0.26M Sucrose	2.08±0.16	0	590.0±50.0	180.0±6.0
0.44M Sucrose	2.55±0.09	0.58±0.08	140.0±70.0	40.0±10.0
0.61M Sucrose	2.50±0.08	0.91±0.09	60.0±5.0	20.0±30.0

Table 4. Effect of sucrose on growth and bud formation of *Bromus inermis* cv. Radison, cultured 6 weeks in 16h photoperiod at 23°C, *in vitro*

Treatment	Tillers and buds per plant	Dormant buds per plant	Fresh Wt, mg per plant	Dry Wt, mg per plant
0.09M Sucrose	0	0	410.0±40.0	130.0±0.8
0.26M Sucrose	1.33±0.11	0	580.0±70.0	180.0±0.2
0.44M Sucrose	1.46±0.10	0.17±0.13	250±15.0	80.0±4.0
0.61M Sucrose	2.35±0.12	1.08±0.09	50.0±3.0	20.0±1.0

Table 5. Effect of sucrose on growth and bud formation of *Dactylis glomerata* cv. Okamidori, cultured 6 weeks in 16h photoperiod at 23°C, *in vitro*

Treatment	Tillers and buds per plant	Dormant buds per plant	Fresh Wt, mg per plant	Dry Wt, mg per plant
0.09M Sucrose	1.83±0.16	0	100.0±10.0	20.0±3.0
0.26M Sucrose	2.00±0.17	0	70.0±8.0	20.0±3.0
0.44M Sucrose	1.12±0.19	0	30.0±4.0	10.0±1.0
0.61M Sucrose	1.22±0.11	0.55±0.08	20.0±2.0	7.0±0.8

Table 6. Effect of sucrose on growth and bud formation of *Poa pratensie* cv.Baron,cultured 6 weeks in 16h photoperiod 23°C, *in vitro*

Treatment	Tillers and buds per plant	Dormant buds per plant	Fresh Wt, mg per plant	Dry Wt, mg per plant
0.09M Sucrose	0.75±0.06	0	132.0±8.0	100.0±2.0
0.26M Sucrose	0.75±0.09	0	81.0±5.0	120.0±8.0
0.44M Sucrose	1.62±0.02	0.87±0.10	21.0±2.0	60.0±6.0
0.61M Sucrose	0.22±0.01	0.22±0.01	-	-

Table 7. Effect of sucrose on growth and bud formation of *Festuca arundinacea* cv. Hokuryo, cultured 6 weeks in 16h photoperiod at 23°C, *in vitro*

Treatment	Tillers and buds per plant	Dormant buds per plant	Fresh Wt, mg per plant	Dry Wt, mg per plant
0.09M Sucrose	0.77±0.13	0	191.0±13.0	33.0±1.0
0.26M Sucrose	0.66±0.08	0	148.0±0.6	38.0±0.6
0.44M Sucrose	0.67±0.12	0.33±0.14	185.0±11.0	62.0±3.0
0.61M Sucrose	0.89±0.10	0.44±0.08	15.0±0.6	6.0±0.3

in tiller production depending on species of grass plants in response to sucrose (Fig. 4). Tillering of *Bromus* and *Festuca* species showed only a slight increase in accordance with raising of sucrose concentration up to 0.61M, but in *Phleum pratense*, and *Dactylis glomerata* tillering is enhanced up to 0.26M sucrose and in *Poa pratense* up to 0.44M.

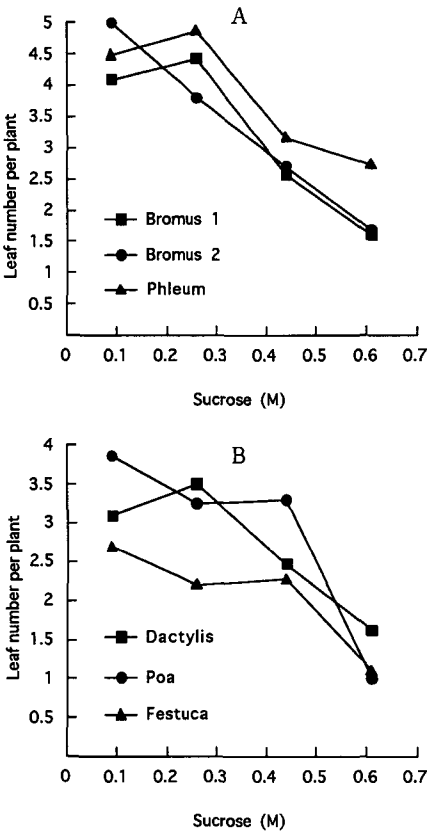

Fig. 3. Effect of sucrose on leaf number of perenial grass species cultured 6 weeks in 16 h photoperiod at 23° C, *in vitro*

Bromus-1: *Bromus inermis* cv Baylor;
Bromus-2: *Bromus inermis* cv Radison;
Phleum: *Phleum pratense* cv Kumpu;
Dactylis: *Dactylis glomerata* cv Okamidori;
Poa: *Poa pratensis* cv Baron;
Festuca: *Festuca arundinacea* cv Hokuryo.

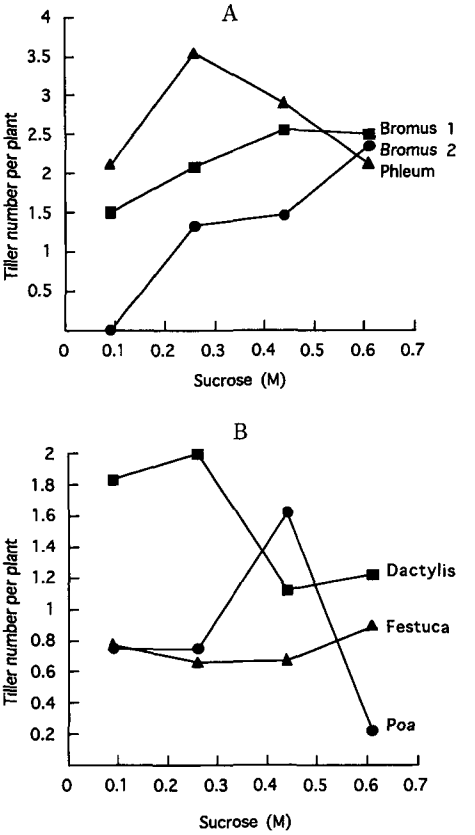

Fig. 4. Effect of sucrose on tiller formation of perenial grass species cultured 6 weeks in 16 h photoperiod at 23 °C, *in vitro*

Bromus-1: *Bromus inermis* cv Baylor;
Bromus-2: *Bromus inermis* cv Radison;
Phleum: *Phleum pratense* cv Kumpu;
Dactylis: *Dactylis glomerata* cv Okamidori;
Poa: *Poa pratensis* cv Baron;
Festuca: *Festuca arundinacea* cv Hokuryo.

In order to search the arrest of vegetative growth followed by enhancing of tillering concomitant with the induction of dormant bud formation in grass species were counted number of axillary buds and tillers formed in cultures of sucrose regime. Tables 1 to 7 show the data of cultured grass species that the dormant bud formation was appeared only when cultures were exposed to relatively high concentration of sucrose, such as 0.44M, while in 0.61M all the plants ceased growing.

Effects of sucrose on dormant bud formation in perennial grass species, *in vitro*

Dormancy of the buds reported in Tables 1 and 3 to 7 was checked by following three methods: 1). transfer of the buds with mother plants onto the basal MS medium and culture them under 16 h photoperiod at 23 °C for another 4 weeks. 2). separate the buds from mother plants entirely, transfer onto the MS basal medium and culture them under 16h photoperiod at 23°C for 4 weeks. 3). determine the metabolic activity of the nonsprouting buds by TTC test.

After 4 weeks culture the buds (Fig. 5), which did not develope into the tiller, were treated by TTC to examine whether the formed buds are metabolically active or not. While all the nonsprouting buds of perennial grass species were not stained pink immediately after incubation of them in TTC solution with succinate at 30°C for 30 minutes, as was not shown by any evidence of the metabolic activity of succinate dehydrogenase in them. This result allowed to conclude the nonsprouting buds were dormant (Fig.6).

The results obtained from the experiments with supplementation of sucrose thus enable to suggest that the osmotic stress caused by high concentration of sucrose was able to induce dormant bud formation in perennial grass.

However, this experiment is not clearly elucidated such problems as dormant buds formed as a response of grass plants to the stress, caused by high concentration of sucrose or formed by affecting of sucrose as a specific metabolite. A decision of these problems was carried out on experiments using mannitol.

Supplementation of mannitol as osmotic reagent on growth of perennial grass species

Mannitol was applied into the medium by following scheme: 0M, 0.17M, 0. 36M, and 0.52M mannitol in addition to 0.09M sucrose, where a sum of the concentrations of mannitol and sucrose was made equal to the concentration of sucrose as in the previous experiment.

The results of the studies on mannitol effects exhibited that an increase in mannitol concentration strikingly reduced the growth rate of all species, far stronger than sucrose. Fresh weight and dry matters (Tabs. 8-13), leaf initation (Fig. 7) and leaf length of all species were shown to decrease intensively according to the increase in mannitol concentration. The data of leaf — length of all

species also were much shorter than those of control. Table 14 has been represented only a result of *Phleum pratense*, as an example.

A study of tillering has demonstrated that those of all species were enhanced by mannitol application, though there was a certain variation in the main peak of tillering among species of grass plants in response to the applied mannitol. For example: tillerings of *Bromus inermis* cv. Baylor, *Dactylis glomerata*, *Poa praten-*

Table 8. Effect of mannitol on growth and bud formation of *Phleum pratense* cv. kumpu, cultured 6 weeks in 16h Photoperiod at 23°C, *in vitro*

Treatment	Tillers and buds per plant	Dormant buds per plant	Fresh Wt, mg per plant	Dry Wt, mg per plant
0.09M Sucrose	0.33±0.15	0	203.0±5.0	54.0±2.0
0.09M Sucrose +0.17M Mannitol	1.22±0.18	0	127.0±3.0	41.9±1.0
0.09M Sucrose +0.35M Mannitol	1.75±0.23	0	100.0±3.0	32.2±0.2
0.09M Sucrose +0.52M Mannitol	3.00±0.27	0	51.0±0.9	15.3±0.4

Table 9. Effect of mannitol on growth and bud formation of *Bromus inermis* cv. Baylor, cultured 6 weeks in 16h photoperiod at 23°C, *in vitro*

Treatment	Tillers and buds per plant	Dormant buds per plant	Fresh Wt, mg per plant	Dry Wt, mg per plant
0.09M Sucrose	0.17±0.05	0	453.0±14.0	75.0±2.0
0.09M Sucrose +0.17M Mannitol	0.83±0.08	0	149.0±6.0	45.0±2.0
0.09M Sucrose +0.35M Mannitol	1.58±0.12	0	49.0±0.8	14.0±3.0
0.09M Sucrose +0.52M Mannitol	2.08±0.01	0	38.0±2.0	9.0±0.2

Table 10. Effect of mannitol on growth and bud formation of *Bromus inermis* cv.Radison, cultured 6 weeks in 16h photoperiod at 23°C, *in vitro*

Treatment	Tillers and buds per plant	Dormant buds per plant	Fresh Wt, mg per plant	Dry Wt, mg per plant
0.09M Sucrose	0	0	286.0±11.0	48.0±2.0
0.09M Sucrose +0.17M Mannitol	1.50±0.17	0	155.0±7.0	44.0±2.0
0.09M Sucrose +0.35M Mannitol	3.17±0.20	0	40.0±2.0	11.0±0.4
0.09M Sucrose +0.52M Mannitol	1.54±0.12	0	33.0±1.0	8.0±0.2

sis, and *Festuca arundinacea* (Fig. 8) were promoted by mannitol application up to 0.26M, on other hand, those in *Bromus inermis* were promoted up to 0.44M, and only the tillering of *Phleum pratense* was specifically continued to increase up to 0.61M mannitol. These results evidenced that effect of mannitol on tillering in

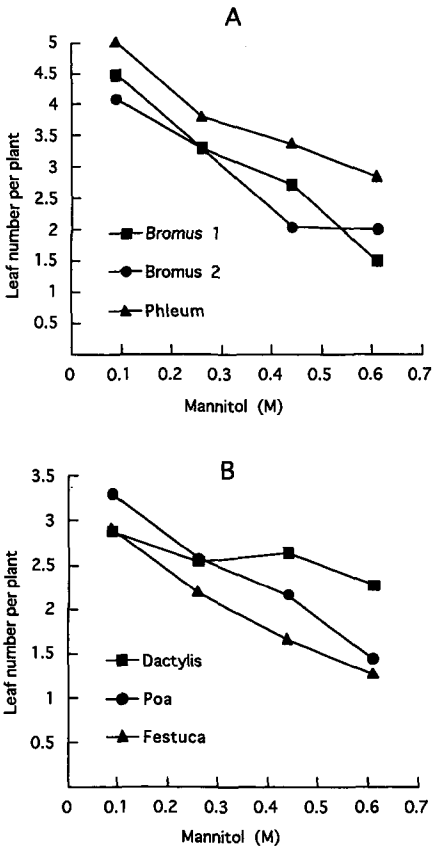

Fig. 7. Effect of mannitol supplemented with 0.06M sucrose on leaf number of perenial grass species cultured 6 weeks in 16 h photoperiod at 23 °C, *in vitro*

Bromus-1: *Bromus inermis* cv Baylor;
Bromus-2: *Bromus inermis* cv Radison;
Phleum: *Phleum pratense* cv Kumpu;
Dactylis: *Dactylis glomerata* cv Okamidori;
Poa: *Poa pratensis* cv Baron;
Festuca: *Festuca arundinacea* cv Hokuryo.

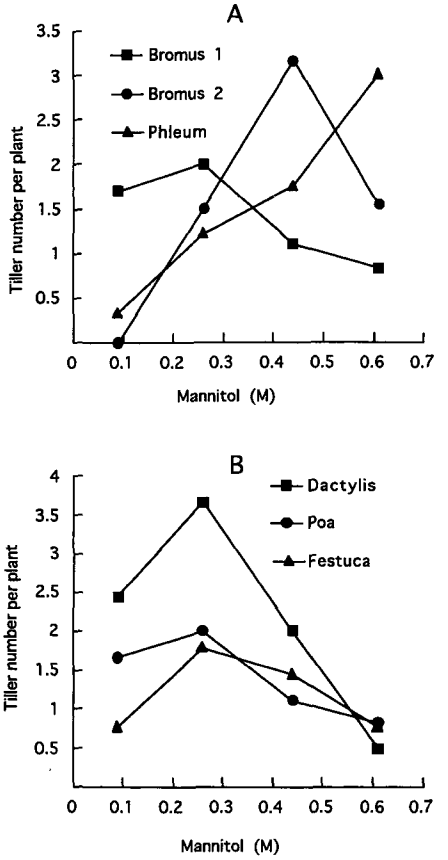

Fig. 8. Effect of mannitol supplemented with 0.06M sucrose on tiller formation of perenial grass species cultured 6 weeks in 16 h photoperiod at 23 °C, *in vitro*

Bromus-1: *Bromus inermis* cv Baylor;
Bromus-2: *Bromus inermis* cv Radison;
Phleum: *Phleum pratense* cv Kumpu;
Dactylis: *Dactylis glomerata* cv Okamidori;
Poa: *Poa pratensis* cv Baron;
Festuca: *Festuca arundinacea* cv Hokuryo.

grass was similar to the effect of sucrose, although variables depended on grass species.

The studies on the tillering response of perennial grass to stress are allowed to divide all grass species of ours investigation into 2 groups: 1. the species with high tillering capacity, such as *Phleum pratense*; 2. the species with low tillering capacity, which involves all other species. The similarities of sucrose, and mannitol-induced retardation of the growth and tillering brought us an attention to analyse dormant bud formation in grass pecies, *in vitro*.

The analysis of effect of mannitol on the induction of dormant bud formation shown in Tables 8 to 13, has revealed that mannitol did not form any type of dormant buds, in contrast to sucrose. This is an important difference between the responses of the application of mannitol and of sucrose to the cultured grass species.

These data could be allowed to suggest that dormant bud induction in perennial grass is not only due to osmotic and water stress caused by mannitol, but also it might be due to a specific stress inducing such high concentration of sucrose as in a case of our study.

Table 11. Effect of mannitol on growth and bud formation of *Dactylis glomerata* cv.Okamidori, cultured weeks in 16h photoperiod at 23°C, *in vitro*

Treatment	Tillers and buds per plant	Dormant buds per plant	Fresh Wt, mg per plant	Dry Wt, mg per plant
0.09M Sucrose	2.44±0.22	0	129.0±11.0	47.0±0.3
0.09M Sucrose +0.17M Mannitol	3.67±0.24	0	137.0±6.0	38.0±2.0
0.09M Sucrose +0.35M Mannitol	2.00±0.12	0	92.0±2.0	29.0±0.6
0.09M Sucrose +0.52M Mannitol	0.52±0.34	0	38.0±4.0	14.0±2.0

Table 12. Effect of mannitol on growth and bud formation of *Poa pratensis* cv. Baron, cultured 6 weeks in 16h photoperiod at 23°C, *in vitro*

Treatment	Tillers and buds per plant	Dormant buds per plant	Fresh Wt, mg per plant	Dry Wt, mg per plant
0.09M Sucrose	1.67±0.11	0	191.0±7.0	46.0±2.0
0.09M Sucrose +0.17M Mannitol	2.00±0.21	0	69.0±2.0	20.0±0.5
0.09M Sucrose +0.35M Mannitol	1.11±0.17	0	25.0±2.0	7.0±0.7
0.09M Sucrose +0.52M Mannitol	0.83±0.22	0	14.0±0.2	5.0±0.2

Table 13. Effect of mannitol on growth and bud formation of *Festuca arundinacea* cv. Hokuyo, cultured 6 weeks in 16h photoperiod at 23°C, *in vitro*

Treatment	Tillers and buds per plant	Dormant buds per plant	Fresh Wt, mg per plant	Dry Wt, mg per plant
0.09M Sucrose	0.78±0.11	0	244.0±2.0	38.0±0.2
0.09M Sucrose +0.17M Mannitol	1.78±0.22	0	117.0±5.0	25.0±0.2
0.09M Sucrose +0.35M Mannitol	1.44±0.11	0	35.0±0.9	10.0±0.3
0.09M Sucrose +0.52M Mannitol	0.78±0.07	0	14.0±0.3	4.0±0.1

Requirement of exogenous ABA for dormant bud formation in *Phleum pratense*

On the basis of above described experiments the third step of our study was to analyse the effect of exogenous ABA application on the induction of dormant bud formation in perennial grass species. The similar responses of grass to the stress factor allowed us to culture only *Phleum pratense* in the experiments with exogenous ABA. This was chosen for several reasons based on the specific feature of this species.

First of them is the relatively high tillering capacity during culture *in vitro*, the most important expression of axillary bud formation in grass plants.

At the next, a germination rate of *Phleum pratense* is also relatively higher (82%) than other species, and it can be grown aseptically with ease in the laboratory. Consequently, the latter is providing one of the excellent experimental systems for the analysis of factors regulating the dormant bud formation in perennial grass.

In the studies were used series of the MS medium, supplemented with 10^{-5}M, 3×10^{-5}M, and 10^{-4}M of ABA, and 0.26M of sucrose for the culture. An addition of 0.26M sucrose to the MS medium is concerned with the fact that this amount of sucrose was the best promotion of the vegetative growth in all species investigated in our experiments, as shown in Tables 1 to 7.

The MS basal medium supplemented with 0.26M sucrose was used as a control medium for this experiment.

The vegetative growth of plants, treated by different concentration of ABA with 0.26M sucrose, was dramatically reduced, — especially leaf initation, leaf length, fresh and dry matters of plants (Tab. 15), whereas tillering and dormant bud formation were increased according to the application of ABA (Fig. 9). Consequently these data could be allowed to assume that the formation of dormant buds required the relatively high level of exogenous ABA, supplemented with 0.26M sucrose in our culture system.

Table 14. Effect of mannitol on leaf growth of *Phleum pratense* cv. Kumpu, cultured 6 weeks in 16h photoperiod at 23°C, *in vitro*

Treatment	Leaf number per plant	Leaf length, cm 1-st	2-nd	3-rd	4-rd	5-th	6-th
0.09M Sucrose	5.0 ±0.13	14.1 ±0.11	4.56 ±0.15	7.28 ±0.29	11.36 ±0.54	12.53 ±0.59	7.84 ±0.43
0.09M Sucrose +0.17M Mannitol	3.79 ±0.16	1.14 ±0.09	2.79 ±0.22	5.29 ±0.43	8.75 ±0.51	8.10 ±0.41	4.42 ±0.19
0.09M Sucrose +0.35M Mannitol	3.37 ±0.79	1.07 ±0.08	2.23 ±0.16	2.52 ±0.28	3.25 ±0.30	2.3 ±0.14	-
0.09M Sucrose +0.52M Mannitol	2.94 ±0.09	0.68 ±0.04	1.09 ±0.07	1.16 ±0.08	2.57 ±0.22	-	-

Table 15. Effect of ABA on growth *Phleum pratense* cv. kumpu cultured 6 weeks in 16h photoperiod at 23°C in MS medium, supplemented with 0.26M sucrose

Treatment	Leaf number per plant	Leaf length, cm 1-st	2-nd	Fresh Wt, mg per Plant	Dry Wt, mg per Plant
0.26M Sucrose +10-5MABA	2.38+0.28	0.92+0.26	1.02+0.15	19.0+0.5	4.3+0.2
0.26M Sucrose +3×10-5MABA	2.26+0.34	0.86+0.35	0.95+0.22	18.0+3.0	5.0+0.8
0.26M Sucrose +10-4MABA	2.15+0.42	0.96+0.13	0.93+0.24	15.0+1.6	5.3+1.0

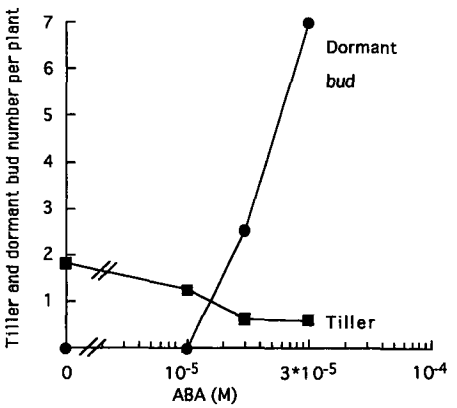

Fig. 9. Effect of ABA on tiller formation and dormant bud formation of *Phleum pratense*, cv Kumpu, cultured 6 weeks in 16 h photoperiod at 23 °C , *in vitro*

Discussion

The cultures of perennial grass species

In natural environment, the induction of dormant structure generally occurs immediately following periods of vigorous growth, when daylength and temperature would still expected to be conductive to growth[15]. This implies that formation of dormant buds is programmed as active events in plant development rather than a simple response to any environmental stimulus. There appears to be an interaction between endogenous and exogenous signals that the efficiency of any single signal is dependent on the balance of the developmental state of the organ and the environment. Therefore all experiments examining of effects of sucrose, mannitol and exogenous ABA were conducted under the constant conditions *i.e.*, under 16 h photoperiod at 23 °C, which were thought to be sufficient for growth of plants, *in vitro*.

Experimental manipulations in studies on vegetative growth response of grass plants to the enviromental stress are usually applied to whole plants. This can hinder progress in understanding the mechanisms of responses of plants cultured, *in vitro*, for at least three reasons. First, complex interactions may occur between organs of the plant. Second, it is difficult to control the effective growth substances, which applied to the whole plant. Third, a whole plant often requires a lot of space and care.

Thus, the understanding of the role of sucrose, mannitol and exogenous ABA in dormant bud formation in grass is necessary to make the system as simple as possible. Therefore, in order to examine the effect of exogenously applied sucrose, mannitol and ABA on development of vegetative growth and on induction of dormant bud in grass plants, all plants were cut at the 0.7-1.0 cm above stem base before the start of experiments. Thereby, all measurements were made comparatively easy, and allowed to reflect the function of root-sourced agents in perennial grass more or less clear.

Effect of sucrose on growth and dormant bud formation in perennial grass

The reduction of growth rate is an integral and essential part of the turion or dormant bud forming response of plants[17,19]. When we affected plants of all species of perennial grass examined by the application of various amounts of sucrose, the growth cessation *i.e.* the reduction and retardation of leaf initation, of leaf enlargement, and concequently fresh and dry matters of newly formed plant mass, was obtained by affecting relatively high concentration of sucrose (more than 0.26M). Thereby, related it the first step of induction of bud dormancy, even the tillering in grass was increased corresponding to the amounts of sucrose.

The above described results enable to predict that increased level of the endogenous ABA in root cells, corresponding to the osmotic stress caused by

higher amounts of sucrose, gave rise to induce formation of dormant buds in grass plants. The similar function of sucrose were reported by Takayama and Misawa[20], Aquettaz *et.al.*[1], and De-Klerk and Gerrits[7]. They were demonstrated that, when the nutritional factors had been examined in lily cultures, the addition of sucrose was only found to influence the establishment of dormancy in *Lilium auratum*, *L. speciosum*, and in 2 cultivars of *L. spectrosum*, while the other changes in medium composition, *i.e.* the type of carbohydrate and the concentration of salts, had no effect on dormancy.

However, in most plants affected by higher amount of sucrose *i.e.* 0.61M,the grass have been revealed senescence of leaves residue and tillers, which could be related to the insufficiency of the root-sourced endogenous ABA in a regulation of osmotic potential of plant organ under a consistent condition during long-termed experiment. In nature, the amount of root-sourced endogenous ABA could be added by ABA produced in leaves, as a result of those ABA accumulation was enough to lead the formation of dormant buds for the growth renewal of grass under favorable conditions.

Effect of mannitol on dormant bud formation in grass species

The grass plants cultured with mannitol gave us an evidence that any growth cessation was not accompanied with the induction of dormant buds. This situation can be explained that osmotic stress is caused, at first, in the root part of plants by mannitol, which is leading to enhance the endogeneous level of ABA and as a result of such process the growth of grass examined was reduced greatly. While the growth cessation observed was not accompanied with dormant bud formation, in which growth cessation caused by mannitol is not sufficient by itself to create more or less stable dormant state.

This result might be linked with a line of mannitol-induced enhancement of ABA in the cultures, whereas plant was not enough sensitive to the latter[7,13] to develop dormancy. Because the extensive ABA accumulation occurred in water stressed tissue, as reported[2,5,21], these could be accompanied by a decreasing sensitivity to ABA in tissue adaptation, while the control of growth was solely a function of endogenous ABA[16].

Effect of exogenous ABA on dormant bud formation in *Phleum pratense*

The results obtained from experiments with exogenous ABA are able to propose that ABA is really controlled vegetative growth of perennial grass, and only a high concentration of ABA, *i.e.* 3×10^{-5} M and 10^{-5} M , supplemented with 0.26M sucrose can induce dormant bud in perennial grass in cultures under growth conditions in long day photoperiod. However, the application of ABA could not exhibit any induction of dormant buds when plant tissue were not readily sensitive to the exogenous applied ABA and also this could be one of the evidences of Strickland-Loeb model described by Trewavas and Jones[22]. The latter is

involved in the fact that plant cells lower their ABA receptor number during development so as it be insensitive to the normal circulating ABA levels, and the well watered and normally grown plants may have lower sensitivity to the exogenously applied ABA. When exogenously applied ABA were supplemented with 0.26M sucrose into the medium of culture, it gave rise to associate with relatively intensive induction of dormant buds. It could be explained by the function of sucrose as a signal substance, which increases ABA receptor number, thereby modified the plant sensitivity to ABA, even though there any clear pathway of this metabolic process were found.

Summary

The dormant bud occurs in perennial grass species that grow regions of temperate climate with cool and chilly season, having finally heavy snow-fall. The environmental control of dormancy and the involvement of hormones in its induction have been little studied.

By employing the aseptic culture system, an attempt has been made to elucidate physiological traits of growth and development of cool-season perennial grass plants of Poaceae, and this relates to depict the hormonal and nutritional conditions favourable for dormant bud formation. The cultures of selected perennial grass species, such as *Bromus inermis*, cv. Baylor, *B. inermis*, cv. Radison, *Phleum pratense*, cv. Kumpu, *Dactylis glomerata*, cv. Okamidori, *Poa pratensis*, cv. Baron and *Festuca arundinacea*, cv. Hokuryo were conducted under the conditions of 16 h photoperiod at 23°C, *in vitro*.

All grass plants, grown from seeds for 5 weeks, multiplied with 1mg/l BA (w/v) for 4 weeks, were clipped at 0.7-1.0cm upper than their root-crown, and the resulting stem bases were used as explants for the studies on dormant bud formation. To analyse the induction of the dormant bud formation, explants were cultured with sucrose (0M, 0.09M, 0.26M, 0.44M, and 0.61M), mannitol (0.17M, 0.35M, 0.52M mannitol, supplemented with 0.09M sucrose), and/or ABA (10^{-5}M, 3×10^{-5}M, and 10^{-4}M, supplemented with 0.26M sucrose).

The results showed that growth cessation and tillering occurred in all cases as supplemented with sucrose, mannitol, and exogenously applied ABA, while dormant bud formation appeared only in plant cultures supplemented with relatively higher concentration of sucrose (0.44M and 0.61M) or 0.26M sucrose with ABA in high levels (3×10^{-5}M and 10^{-4}M).

In addition, the analysis of TTC staining for succinate dehydrogenase activity of formed buds has been exhibited by a substantial reduction of metabolic activity, due to the increased amount of sucrose and ABA in the medium.

These results suggest that ABA plays a key role in the induction and development of dormant bud in perennial grass species under aseptic cultures.

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References

1. Aquettaz, P, Paffen, A, Delvallee, I, Van der Linde, P, and De Klerk, GJ. : The development of dormancy in bulblets of Lilium speciosum generated in vitro. 1. The effects of culture conditions. *Plant Cell Tissue and Organ Culture* **22** : 167-172. 1990.
2. Beardsell, M F and Cohen, D.: Relationships between leaf water status, abscisic acid levels and stomatal resistance in maize and sorghum. *Plant Physiol.* 56 : 207-212. 1975.
3. Busso, CA, Mueller, RJ and Richards, JH.: Effect of drought and defoliation on bud viability in two Caespitose Grasses. *Annals of Bot.* **63** : 477-485. 1989.
4. Busso, CA, Boo, RM and Pelaez, DV.: Fire effects on bud viability and growth of Stipa tenius in Semiarid Argentina. *Annals of Botany*, **71** : 377-381. 1993.
5. Cornish, K and Radin, JW.: From metabolism to organism : an integrative view of water stress emphasising abscisic acid. In: Katterman FJ. ed., Environmental injury to plants. *Academic Press, New York*, 89-112. 1990.
6. Culvenor, RA.: Effect of cutting during reproductive development on the regrowth and regenerative capacity of theperennial grass, *Phalaris aquatica* L., in a controlled environment. Annals of Bot. **72** : 559-568. 1993.
7. De Klerk, GJ and Gerrits, MM.: Dormancy in tissue-cultured lily bulblets and apple shoots. In: Lang GA ed.Plant Dormancy : Physiology, Biochemistry and Molecular Biology. *CAB International, London.* 115-131. 1996.
8. Hume, DE.: Leaf and tiller production of prairie grass (*Bromus willddenowii* Kunth) and two *ryegrass (Lolium)* species. *Annals of Bot.* **67** : 111-121. 1991.
9. Hume, DE.: Effect of cutting on production and tillering in prairie grass (*Bromus willdenowii* Kunth) compared with two ryegrass (*Lolium*) species. 1. Vegetative plants. *Annals of Bot.* **67**: 533-541. 1991.
10. Hume, DE.: Effect of cutting on production and tillering in prairie grass (*Bromus willdenowii* Kunth) compared with two ryegrass (*Lolium*) species. 2. Reproductiveplants. —*Annals of Botany*, **68** : 1-11. 1991.
11. Hyder, DN.: Defoliation in relation to vegetative growth. In: Younger VB, McKell CM, eds. Biology and utilization of grasses. *New York: Academic press*, 304-317. 1972.
12. Jewiss, OR.: Tillering in grasses—its significance and control. *Jour. of the British Grassl. Soc.* **27** : 65-82. 1972.
13. Kim, Y-K, Howard, BH and Quinlan, JD.: Apparent ABA—induced inhibition of the lower

laterals of one—year old cherry trees. *Jour. Hort. Sci.* **59** : 35-44. 1994.
14. Murashige, T and Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15** : 473-475. 1962.
15. Powell, LE.: The hormonal control of bud and seed dormancy in woody plants. In: Davies PJ, ed., Plant hormones and their role in plant growth and development.*Kluwer, Dordrecht.* 539-552. 1988.
16. Saab, IN, Sharp, RE, Pritchard, J and Voetburg, GS.: Increased endogenous abscisic acid maintains primary root growth and inhibits shoot growth of maize seedlings at law water potential. *Plant physiol.* **93** : 1329-1336. 1990.
17. Smart, CC and Trewavas, AJ.: Abscisic acid-induced turion formation in *Spirodela polyrrhiza* L. 1. Production and development of the turion. *Plant Cell Environ.* **6** : 507-514.1983.
18. Steponkus, DE and Lanphear, FO.: Refinement of the triphenyl tetrazolium chloride method of determining cold injury. *Plant Physiol.* **42** : 1423-1426. 1967.
19. Stewart, GR.: Abscisic acid and morphogenesis in *Lemna polyrrhiza* L. *Nature*, **221** : 61-62. 1969.
20. Takayama, S and Misawa, M.: Differentiation in *Lilium bulbscales* grown in vitro: effects of activated charcoal, physiological age of bulbs and sucrose concentration on differentiation and scale leaf formation in vitro. *Physiol. Planta.* **48** : 121-125. 1980.
21. Trewavas, AJ.: Sensitivity and sensory adaptation ingrowth substance responses. In: Houd GV, Lenton JR, Jackson MB and Atkin RK. eds. Hormone action in plant development. *Butterworths, London,* 19-39. 1987.
22. Trewavas, AJ and Jones, HG.: An assessment of the role of ABA in plant development. In: Davies WJ and Jones GH. eds. Abscisic acid : Physiology and Biochemistry. *Bios Scientific Publishers, Oxford,* 169-188. 1991.
23. Tuomi, J, Nilsson, P and Astrom, M.: Plant compensatory responses: Bud dormancy as an adaptation to herbyvory. —*Ecology*, **75**(5) : 1429-1436. 1994.
24. Xu, Z-J, Fujino, K and Kikuta, Y.: Regeneration via somatic embryogenesis and/or organogenesis in callus from mature seeds of *Echinochloa oryzycola* Vasing. *Japanese Jour. Crop Sci.*, **62** : 614-620. 1993.

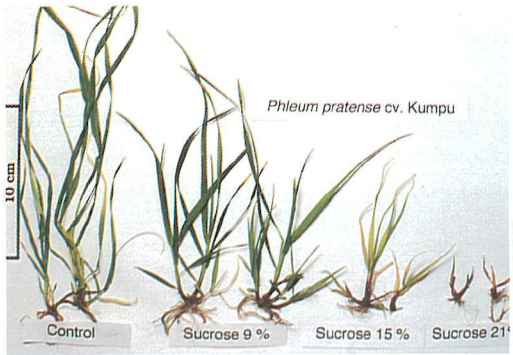

Fig. 2. Effect of sucrose on growth of *Phleum pratense*, cv Kumpu, cultured 6 weeks in 16 h photoperiod at 23 °C, *in vitro*

Fig. 5. Dormant buds of Phleum pratense cv Kumpu induced in 16 h photoperiod at 23 °C, *in vitro*

Fig. 6. The longitudinal section of dormant buds before (A) and after (B) incubation with TTC. Dormant buds were not stained pink when succinate was used as substrate.