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Development of *in vitro* shoot cultures of strawberry (*Fragaria* × *ananassa* Duch.) 'Senga Sengana' and 'Elsanta' under the influence of high doses of gibberellic acid

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ABSTRACT

In this study, the influence of gibberellic acid (GA_3) on strawberry *in vitro* shoot culture growth and development was investigated. 'Senga Sengana' and 'Elsanta' clones were grown on the medium recommended by Boxus (1999), supplemented with BA (0.5 mg dm^{-3}), IBA (0.1 mg dm^{-3}), glucose (40.0 g dm^{-3}) and GA_3 (0.1 – control, 1.0, 2.0, 5.0, 10.0 mg dm^{-3}). In general, gibberellic acid improved axillary shoot elongation and reduced the growth of callus as well as the formation of roots and the development of adventitious shoots. GA_3 applied at a concentration of 1.0-2.0 mg dm^{-3} significantly increased the number of axillary shoots (mainly crown shoots), whereas under higher (5.0-10.0 mg dm^{-3}) doses it stimulated the development of runners. It seems that a new method of strawberry micropropagation based on the multiplication of axillary crowns and runners,

which could also reduce the risk of domination of *in vitro* cultures with adventitious shoots, might be elaborated.

Abbreviations:

BA – 6-benzylaminopurine
IBA – indole-3-butyric acid
GA_3 – gibberellic acid.

INTRODUCTION

The micropropagation of strawberry was achieved more than 30 years ago (Boxus 1974), and now it seems to be a routine task in many commercial laboratories. Numerous studies have been published regarding field behaviour of micropropagated strawberry. Sometimes, such plants more or less often exhibited characteristics such as dwarfism, chlorosis or white striping of leaves, multi-apexing and stem fasciations, intensified vigour, hyperrunnering and abnormal flowering (hyperflowering), accompanied by increased production of smaller fruits (Anderson et al. 1982, Boxus et al. 2000, Graham 2005). Some concern has been expressed about the genetic stability of micropropagated plants (Dijkstra 1990, Graham 2005). Therefore, the technique declined in popularity (Zimmerman 1991) and was rejected or limited by law in some countries (Boxus 1999).

Jemmalli et al. (1992 and 1994) found that adventitious (stipular) shoots occurred spontaneously, and were common in strawberry *in vitro* shoot cultures. They also revealed the relationship between adventitious shoots arisen *in vitro* and plant hyperflowering. Boxus (1999) underlined the risks introduced by adventitious regeneration in mass propagation systems of strawberry. To avoid this, using a decreasing concentration of BA, shortening the subculture period, and limiting the number of subcultures is recommended (Jemmalli et al. 1992, Graham 2005). Unfortunately, such practices increase the costs of micropropagation and do not guarantee the propagation of strawberry exclusively through axillary shoots (without adventitious ones).

Many years ago Murashige (1964) found that one of the effects of GA_3 application was the retardation of adventitious shoot development in tobacco *in vitro* cultures. Gibberellic acid is used in the micropropagation of strawberry. In general, it is applied at the concentration of 0.1 mg dm^{-3} at the proliferation stage (Boxus 1974 and 1999, Litwińczuk and Zubel 2005). Only a few authors (Badawi et al. 1990, Kaur et al. 2005, Kanika et al. 2006) used GA_3 in higher doses (1-2 mg dm^{-3}). The influence of gibberellic acid on strawberry *in vitro* cultures was studied by Waithaka et al. (1980) and Zatyko et al. (1989). They found that GA_3 (10-20 mg dm^{-3}) improved strawberry shoot elongation and stimulated development of runners *in vitro*. None of the aforementioned authors reported whether GA_3 had modified

the formation and growth of adventitious (stipular) shoots *in vitro*, possibly because of the fact that the studies of Jemmali et al. (1992 and 1994) were published some years later. Thus, the influence of gibberellic acid used in various concentrations on the development of axillary and adventitious shoots was investigated again.

MATERIAL AND METHODS

The experiment was carried out on *in vitro* cultures of two strawberry (*Fragaria* × *ananassa* Duch.) cultivars, 'Senga Sengana' and 'Elsanta'. The influence of gibberellic acid (GA_3 0.1, 5.0, 10.0 mg dm^{-3}) was investigated through three subsequent 4-5-week long subcultures. In the first subculture, GA_3 was also used in 1.0 and 2.0 mg dm^{-3} concentrations. Gibberellic acid was added to the media before autoclaving (121ºC, 15 min). The cultures were grown under cool-white fluorescent light (OSRAM) at 38.9 µmol m^{-2} s^{-1} PPFD and 26 ± 1ºC in Erlenmayer flasks (100 cm^{-3}) filled with medium (30.0 cm^{-3}) and closed with aluminium foil. The basic medium (control) recommended by Boxus (1999), supplemented with BA (0.5 mg dm^{-3}), GA_3 (0.1 mg dm^{-3}), IBA (0.1 mg dm^{-3}), glucose (40.0 g dm^{-3}) and Gibco + Kobe I agars (3.0 + 3.0 g dm^{-3}), was applied. Axillary crown shoots (rosettes, R) were placed on control medium (GA_3 0.1 mg dm^{-3}) and the tips of the elongated crown shoots (ST) or the tips of the runners (RT) were used on other media (GA_3 1.0-10.0 mg dm^{-3}). Explants were prepared from preliminary cultures grown on media supplemented with the appropriate GA_3 dose. Each combination was represented by five flasks in the first subculture and 10 flasks in the second and third ones. The replication was one flask containing six cultures grown on the basic medium. Thus, each combination was represented by at least 30 cultures grown on the basic medium in each subculture. The following data were collected at the end of each subculture: number of crown shoots, number of runners, number of rooted cultures, callus diameter. They were then subjected to the ANOVA and LSD mean separation test at $p = 0.05$ significance level using Statgraphics 4.2 computer software.

RESULTS

A distinct relationship between GA_3 concentration and the development of strawberry *in vitro* shoot cultures was found. Explants placed on the control medium (GA_3 0.1 mg dm^{-3}) started to develop both axillary and adventitious (stipular) shoots early (Fig. 1). Adventitious shoots arose close to axillary shoots. They were difficult to distinguish at the end of the subculture as a relatively big callus covered the culture base (Fig. 2). Many control cultures developed roots

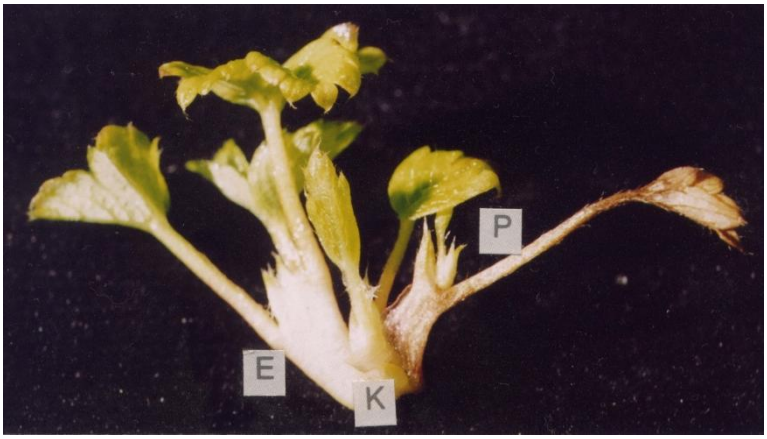

Figure 1. Young *in vitro* culture of strawberry 'Elsanta'; E – initial explant (crown, rosette), K – new axillary crown, P – new adventitious (stipular) shoot

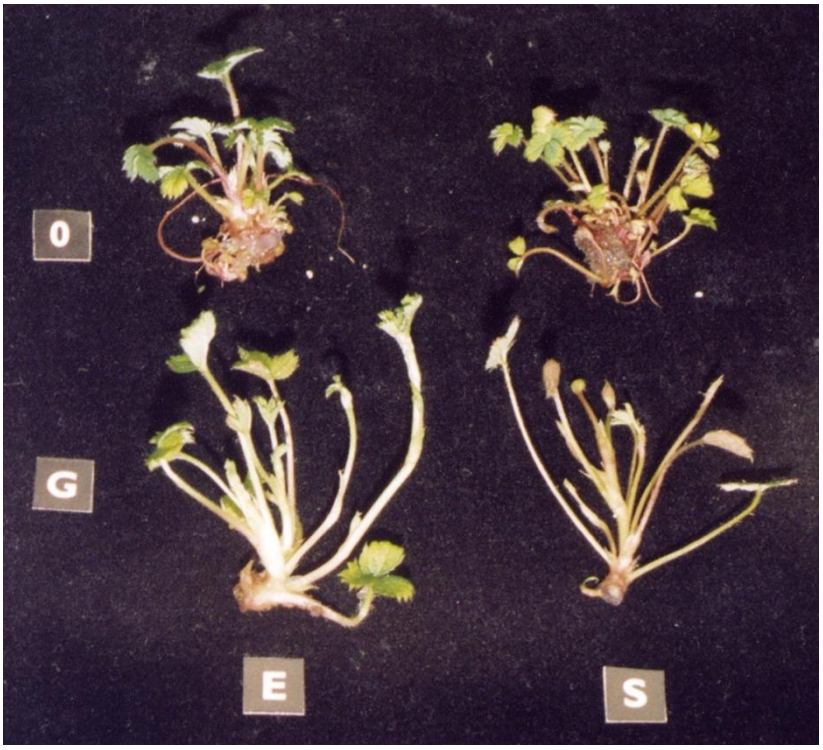

Figure 2. *In vitro* cultures of strawberry 'Elsanta' (E) and 'Senga Sengana' (S) obtained on media supplemented with GA_3 (O – 0.1 mg dm^{-3}, control; G – 10.0 mg dm^{-3})

Table 1. Development of strawberry 'Senga Sengana' *in vitro* cultures on medium supplemented with gibberellic acid

Passage	GA_3 (mg dm^{-3})	Explant	Number of axillary rosettes	Number of axillary runners	Total number of axillary shoots	Ratio of axillary shoots in culture (%)	Number of adventitious rosettes	Number of adventitious runners	Total number of adventitious shoots	Total number of shoots	Callus size (mm)	Number of cultures developing roots (%)
I	0.1	R[1] (control)	3.6 b[2]	0.0 a	3.6 a	55.5 a	nd[3]	nd	3.1 b	6.7 ab	4.8 d	68.0 d
	1.0	ST	6.2 c	0.3 a	6.5 cd	87.4 b	nd	nd	1.1 a	7.5 b	4.3 cd	33.3 c
	2.0	ST	5.6 c	1.6 b	7.1 d	92.4 b	nd	nd	0.7 a	7.8 b	3.9 c	35.0 c
	5.0	ST	3.3 b	3.4 d	6.7 cd	85.0 b	nd	nd	1.4 a	8.1 b	3.2 b	29.2 bc
	5.0	RT	3.5 b	2.0 b	5.5 bc	84.6 b	nd	nd	1.1 a	6.6 ab	2.6 a	4.8 a
	10.0	ST	2.3 a	2.8 cd	5.1 b	92.2 b	nd	nd	0.5 a	5.6 a	2.8 ab	7.7 ab
	10.0	RT	1.9 a	2.2 bc	4.1 ab	85.1 b	nd	nd	1.3 a	5.4 a	2.7 a	10.0 ab
II	0.1	R (control)	3.9 d	0.0 a	3.9 a	73.2 a	2.1 c	0.0 a	2.1 a	6.0 a	8.5 d	30.0 b
	5.0	ST	3.3 cd	3.4 b	6.7 b	85.9 b	0.7 ab	0.8 b	1.4 a	8.1 b	5.7 c	0.0 a
	5.0	RT	2.5 ab	3.7 b	6.1 b	93.0 b	0.3 ab	0.2 ab	0.5 a	6.6 ab	4.6 b	0.0 a
	10.0	ST	3.0 bc	5.9 c	8.9 c	85.3 b	1.0 b	0.8 b	1.8 a	10.7 c	4.9 b	0.0 a
	10.0	RT	1.7 a	4.1 b	5.8 b	91.8 b	0.1 a	1.0 b	1.1 a	7.0 ab	3.5 a	0.0 a
III	0.1	R (control)	2.8 d	0.0 a	2.8 a	59.7 a	2.4 b	0.0 a	2.4 c	5.2 c	5.4 c	81.5 b
	5.0	ST	1.8 c	2.1 c	3.8 b	89.6 bc	0.2 a	0.8 bc	1.0 b	4.8 bc	3.1 b	0.0 a
	5.0	RT	1.8 c	1.4 b	3.2 ab	85.2 b	0.1 a	1.1 c	1.2 b	4.4 abc	2.4 a	0.0 a
	10.0	ST	1.4 b	2.3 c	3.7 b	96.6 d	0.1 a	0.2 a	0.3 a	3.9 ab	2.2 a	0.0 a
	10.0	RT	0.9 a	1.8 bc	2.8 a	95.8 cd	0.1 a	0.3 ab	0.4 ab	3.1 a	1.9 a	0.0 a
Mean	0.1	R (control)	3.4	0.0	3.4	62.8	1.5	0.0	2.5	6.0	6.2	59.8
	5.0	ST	2.8	3.0	5.7	86.8	0.3	0.5	1.3	7.0	4.0	9.7
	5.0	RT	2.6	2.4	4.9	87.6	0.1	0.4	0.9	5.9	3.2	1.6
	10.0	ST	2.2	3.7	5.9	91.4	0.4	0.3	0.9	6.7	3.3	2.6
	10.0	RT	1.5	2.7	4.2	90.9	0.1	0.4	0.9	5.2	2.7	3.3

[1] R – axillary crown shoots (rosettes), ST – tips of elongated crown shoots, RT – tips of runners
[2] Means followed by different letters within a column are significantly different at p = 0.05
[3] nd – not determined

Table 2. Development of strawberry 'Elsanta' *in vitro* cultures on medium supplemented with gibberellic acid

Passage	GA_3 (mg dm^{-3})	Explant	Number of axillary rosettes	Number of axillary runners	Total number of axillary shoots	Ratio of axillary shoots in culture (%)	Number of adventitious rosettes	Number of adventitious runners	Total number of adventitious shoots	Total number of shoots	Callus size (mm)	Number of cultures developing roots (%)
I	0.1	R[1] (control)	2.7 a[2]	0.0 a	2.7 a	63.3 a	nd[3]	nd	2.0 bc	4.7 a	3.7 d	15.4 a
	1.0	ST	4.3 b	0.5 ab	4.8 b	74.7 ab	nd	nd	2.9 c	7.7 c	3.9 d	5.0 a
	2.0	ST	3.8 b	0.9 b	4.7 b	80.0 bc	nd	nd	2.0 bc	6.7 bc	2.5 bc	10.5 a
	5.0	ST	2.5 a	1.9 c	4.4 b	92.1 cd	nd	nd	0.6 ab	5.0 ab	2.4 bc	4.2 a
	5.0	RT	2.4 a	2.2 cd	4.6 b	94.2 cd	nd	nd	0.3 a	4.9 ab	2.7 c	0.0 a
	10.0	ST	1.9 a	2.5 cd	4.4 b	92.6 cd	nd	nd	0.8 ab	5.2 ab	1.7 a	0.0 a
	10.0	RT	2.1 a	2.7 d	4.9 b	96.3 d	nd	nd	0.2 a	5.1 ab	2.1 ab	0.0 a
II	0.0	R (control)	3.4 c	0.0 a	3.4 ab	78.5 a	1.2 b	0.0 a	1.2 a	4.6 a	5.8 d	0.0 a
	5.0	ST	1.8 a	2.2 c	4.0 b	92.1 b	0.6 ab	0.1 a	0.7 a	4.7 a	3.4 c	0.0 a
	5.0	RT	2.7 b	1.6 b	4.2 b	91.0 b	0.3 a	0.2 a	0.5 a	4.7 a	2.8 bc	0.0 a
	10.0	ST	1.5 a	2.3 c	3.8 ab	94.5 b	0.4 a	0.0 a	0.4 a	4.2 a	2.8 b	0.0 a
	10.0	RT	1.6 a	1.4 b	3.0 a	95.0 b	0.3 a	0.1 a	0.5 a	3.5 a	1.9 a	0.0 a
III	0.1	R (control)	3.1 b	0.0 a	3.1 ab	80.4 a	1.2 b	0.0 a	1.2 b	4.3 b	4.1 d	12.2 b
	5.0	ST	1.4 a	2.0 c	3.4 b	98.8 b	0.1 a	0.1 a	0.1 a	3.5 a	2.1 c	0.0 a
	5.0	RT	1.6 a	1.2 b	2.8 ab	90.4 b	0.5 a	0.1 a	0.7 ab	3.5 a	1.9 bc	0.0 a
	10.0	ST	1.4 a	2.0 c	3.4 b	94.6 b	0.2 a	0.0 a	0.2 a	3.6 ab	1.7 ab	0.0 a
	10.0	RT	1.4 a	1.1 b	2.5 a	94.8 b	0.2 a	0.1 a	0.3 a	2.8 a	1.3 a	0.0 a
Mean	0.1	R (control)	3.1	0.0	3.1	74.1	0.8	0.0	1.5	4.5	4.5	9.2
	5.0	ST	1.9	2.0	3.9	94.3	0.2	0.1	0.5	4.4	2.6	1.4
	5.0	RT	2.2	1.7	3.9	91.9	0.3	0.1	0.5	4.4	2.5	0.0
	10.0	ST	1.6	2.3	3.9	93.9	0.2	0.0	0.5	4.3	2.1	0.0
	10.0	RT	1.7	1.7	3.5	95.4	0.2	0.1	0.3	3.8	1.8	0.0

[1, 2, 3] Explanations: see Table 1

from the callus (Fig. 2, Tabs 1 and 2). The appearance of strawberry *in vitro* cultures changed gradually as the GA_3 concentration increased. The elongation of crown shoots as well as runner development was observed (Fig. 2). On the other hand, the growth of callus and roots was inhibited (Fig. 2, Tabs 1 and 2). Gibberellic acid applied in a 1.0-2.0 mg dm^{-3} concentration significantly increased the number of axillary shoots, mainly crown shoots (Tabs 1 and 2). The proliferation of axillary crowns was worsened and the development of runners was stimulated under higher (5.0-10.0 mg dm^{-3}) doses of GA_3 (Tabs 1 and 2). However, many runners (about 40%) were thin and poorly developed. Generally, the total number of axillary shoots increased compared to the control, especially when crown tips were used as explants (Tabs 1 and 2). Gibberellic acid used in higher concentration (minimum 1.0 mg dm^{-3} and 5.0 mg dm^{-3} for 'Senga Sengana' and 'Elsanta', respectively) retarded the formation of adventitious shoots. As a result, the proportion between axillary and adventitious shoots in the culture was significantly higher while compared to the control (Tabs 1 and 2). The shoot tips of elongated crowns were better explants than the runner tips, as they produced more axillary shoots (Tabs 1 and 2). Similar results relevant to the application of GA_3 were obtained for 'Kama', 'Dukat', and 'Kent' *in vitro* cultures (data not presented because the culture sample was too small – under 20 per treatment).

DISCUSSION

The response of *in vitro* cultures of strawberry to high doses of GA_3 included, among others, the elongation of crown shoots and the stimulation of runnering, which was described earlier (Waithaka et al. 1980, Zatyko et al. 1989). Nonetheless, the authors did not mention whether GA_3 influenced shoot proliferation. Only Fouad et al. (1991) informed that explants treated with GA_3 (3.46 mg dm^{-3}) produced single crown cultures. However, they applied filter-sterilised gibberellin and did not use it concurrently with cytokinin. The aforementioned authors (Waithaka et al. 1980, Zatyko et al. 1989, Fouad et al. 1991) did not describe the influence of gibberellic acid on the formation and growth of adventitious (stipular) shoots. In the present study, GA_3 was found to improve the elongation of axillary shoots and stimulate the development of runners *in vitro*. In general, gibberellic acid also improved axillary shoot proliferation. However, it was mainly due to the increased development of runners *in vitro*, as the proliferation of axillary crowns was reduced. The present study showed that gibberellic acid inhibited the development of adventitious shoots. Additionally, thanks to the retardation of basal callus as well as the elongation of internodes and leaf petioles, the adventitious shoots were easier to identify and eliminate during the culture subdivision and transfer of explants onto a fresh medium. Thus, the risk

of domination of *in vitro* cultures with adventitious shoots was reduced. It could favour the longer micropropagation of strawberry through a greater than recommended (10) number of subcultures (Boxus 1999). It is possible that the phenomenon of hyperflowering and reduced fruit size observed among micropropagated plants could then be limited as it is caused by unknowing propagation *in vitro* through adventitious shoots (Jemmali et al. 1992 and 1994). Application of GA_3 should also make the preservation of strawberry germplasm easier. It should be underlined that gibberellic acid did not completely inhibit the development of adventitious shoots. Therefore, strawberry should be propagated through single shoots, not by small tufts of buds as Boxus (1999) recommended.

Some field trials showed that hyperflowering is reduced in subsequent conventional runner generations of micropropagated strawberry plants (Swartz and Lindstrom 1986). Litwińczuk (2004) found that hyperflowering and hyperrunnering could even be reduced by propagation through runners appearing at the end of the adaptation stage of strawberry plantlets. Thus it is possible that the same result may be obtained when plants will be multiplied through runners *in vitro*. The present study showed that strawberry could be propagated *in vitro* by runners, although this method is less efficient than propagation by crown tips and should be improved. It should also be checked whether gibberellic acid applied in high concentrations during micropropagation affects field performance of strawberry. Anderson et al. (1982) found that GA_3 reduced the frequency of multi-apexed plants. However, they used gibberellin at a low concentration (0.1 mg dm^{-3}). Waithaka et al. (1980) and Zatyko et al. (1989) did not mention whether they evaluated the behaviour of micropropagated plants. Only Fouad et al. (1991) informed that gibberellin enhanced juvenile characteristics such as increased plant runnering and delayed flowering, among others. On the other hand, *in vitro* GA_3 treated plants produced fewer inflorescences than the control. However, experiments were carried out with the 'Fern' (*Fragaria* × *ananassa* Duch. × *Fr. virginiana* Duch. ssp. *glauca*) clone on a small sample (10 plants per treatment) in the greenhouse. Thus, at present there is not enough information to evaluate the usefulness of micropropagation of strawberry based on the application of high doses of gibberellic acid.

CONCLUSIONS

As gibberellic acid applied in doses higher than usually used (0.1 mg dm^{-3}) stimulates the proliferation of axillary crown shoots or runners and concurrently reduces the growth of callus as well as the formation of roots and the development of adventitious shoots, a new method of strawberry micropropagation, which lowers the risk of domination of in vitro cultures with adventitious shoots, might be elaborated.

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ROZWÓJ KULTUR *IN VITRO* TRUSKAWKI (*FRAGARIA* × *ANANASSA* DUCH.) 'SENGA SENGANA' I 'ELSANTA' POD WPŁYWEM WYSOKICH DAWEK KWASU GIBERELOWEGO

S t r e s z c z e n i e: W doświadczeniu badano wpływ kwasu giberelowego (GA_3) na wzrost i rozwój pędowych kultur *in vitro* truskawki. Klony 'Senga Sengana' i 'Elsanta' były prowadzone na pożywce Boxusa (1999) uzupełnionej o BA (0,5 mg dm^{-3}), IBA (0,1 mg dm^{-3}), glukozę (40,0 g dm^{-3}) i GA_3 (0,1 – kontrola: 1,0; 2,0; 5,0; 10,0 mg dm^{-3}). Kwas giberelowy pobudzał elongację pędów kątowych i osłabiał wzrost kalusa oraz tworzenie korzeni i pędów przybyszowych. Użyty w stężeniu 1,0-2,0 mg dm^{-3} zwiększał proliferację rozet kątowych, zaś w dawkach wyższych (5,0-10,0 mg dm^{-3}) stymulował rozwój rozłogów *in vitro*. Umożliwia to opracowanie nowej metody mikrorozmnażania truskawki za pomocą rozet i rozłogów kątowych, która jednocześnie zmniejszy ryzyko opanowywania kultur przez pędy pochodzenia przybyszowego.

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