JUNE YELLOWS OF STRAWBERRY

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Consists of two parts:

Part 1: Two papers to be individually submitted for publication concerning major experiments.

Part 2: Other experiments

PRODUCTION OF JUNE YELLOWS OF STRAWBERRY (*FRAGARIA ANANASSA* DUCH.) *IN VITRO*

by

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ABSTRACT

June Yellows affected and unaffected clones of strawberry cultivar Cambridge Favourite were grown in vitro and maintained at 4°C, 13°C and 22°C for three months. June Yellows symptoms were produced chiefly by the affected clones at the two lower temperatures. Material from 4°C and 13°C was divided, half returned to the original maintenance temperature, the other half moved to conditions of 22°C for 9 weeks. The number of plants with symptoms continued to increase at 4°C; a variety of responses emerged from the other treatments. Reasons underlying these observations are discussed.

INTRODUCTION

June Yellows is a chlorotic disorder of strawberries which can cause a 50% yield loss once it appears in a crop (Wilson, 1955). It has been reported in the UK (Reid, 1951), the USA, Canada and Europe (Klinkenberg, 1949; Bovey, 1952). In Scotland the symptoms appear in late April and early May, with the onset of spring conditions when the new leaves emerge marked with varying degrees of chlorosis which range from a mild fleck, through chlorotic patches and streaks, to total chlorosis which is often accompanied by distortion of the leaf. Symptoms fade during the summer months when mildly affected plants resume a normal appearance before symptoms reappear in a less obvious form during the autumn (Plakidas, 1932).

June Yellows is not caused by a nutrient deficiency (Morris et al, 1944) and does not seem to be infectious since all attempts to reproduce the ailment in healthy plants by grafting, transmission of sap, etc (Plakidas, 1932; Guba, 1932, 1933) have failed, although it is possible that infection was achieved but that symptom expression was not induced. Susceptibility to the condition appears to be a heritable characteristic (Anderson, 1935), since successful transmission occurs only by going through seed and all affected cultivars identified thus far share common ancestry with Howard 17 (Premier) and/or Blakemore. Inheritance studies suggest that June Yellows may be

caused by a rogue plasmagene (Williams, 1955) although there is conflicting evidence as to whether inheritance is maternal (McWhirter, 1955), paternal (Williams, 1955; Wills, 1962) or bi-parental (Hedley Williams, 1954). Alternatively the disorder may be caused by a very closely adapted virus (Jones et al, 1986a and b) or mycoplasma-like organism (Huhtanen, 1971) that has developed the ability to be transmitted via the seed to the next generation (Lister, 1960).

Susceptibility to June Yellows is not immediately apparent within a cultivar, and the delay between cultivar release and first symptom expression, up to 10-30 years later (Braak, 1955), may have economic implications for the strawberry industry in a small country such as Scotland which cultivates a particular market-niche and has a heavy dependency on a very small number of varieties. Once symptoms begin to appear, the condition of the plant deteriorates with each passing season, usually resulting in death within 3 years. First symptoms appear simultaneously within a variety across a country; no affected plants have been known to recover (Demaree and Darrow, 1937) although remission of symptoms occurs. To date, moderate control has been achieved by the use in breeding programmes of "green" clones, ie individual lines that have not shown any yellowing although they may come from susceptible varieties.

Fungal and viral infections have caused major problems in strawberry farming in the past. In Scotland these have been combatted, for the most part, by the widespread use of disease free stock produced in commercial quantities by micropropagation. It was hoped that an in vitro test for June Yellows could be developed so that stock produced by such techniques could be screened for susceptibility before release to growers (Reid, 1952-55).

MATERIALS

Source, Culture and Maintenance of Parental Material

Seven "green" NSA clones of Cambridge Favourite, 1A, 3A, 3B, 3C, 3D, 8A and 8B, which had never exhibited June Yellows symptoms were provided by Brogdale EHS

as pot-grown plants. Runners of two "yellow" clones of Cambridge Favourite, Y and YM, were obtained from plants known to be suffering from the disorder while in a multi-centre trial run by the East of Scotland College of Agriculture at Castle Huntly. Affected plants of another Cambridge Favourite "yellow" clone, FY, were supplied from the ESCA field trials in 1987. After initiation both "green" and "yellow" explants were green when put into experiments. (Clone 8A has subsequently been eliminated from NSA mother stocks at East Malling by the NFT Strawberry Panel after developing transient yellows (Posnette, 1955), however, it is maintained at ESCA for research purposes.)

Meristems 0.5-1.0 mm long were excised from runners of these plants under sterile conditions and placed on either M & S or Boxus meristem initiation medium (see Methodology). It is advantageous to culture the meristems immediately without prior sterilisation with Tween 20 (polyoxyethylene sorbitan monolaurate) or similar substances, which tend to produce high death rates. Indeed, meristems are usually sterile when removed from their covering tissue, and very few are lost through infection when placed directly onto the culture medium. After five weeks the explants were transferred to the appropriate proliferation medium. The resulting cultures were maintained as stock cultures under conditions of 1-2 klx illumination for 16 hours per day at 22°C were subcultured onto fresh M & S shoot proliferation medium every 6-10 weeks, depending upon their rate of growth.

METHODOLOGY

Prior to their entry into the in vitro system observational studies were carried out on the mother plants of the seven NSA Cambridge Favourite clones to determine whether any major differences existed in vivo which could confuse interpretation of observations made at a later date in vitro. The following vegetative and morphological measurements were taken:-

(i) petiole length

(ii) petiole hair length

(iii) central leaflet length and width

(iv) number of serrations on the left side of the central leaflet

(v) stomatal count (the abaxial surface of the leaves was painted with colourless nail varnish which was allowed to dry, then peeled off and examined under the microscope)

(vi) chloroplast count (using the method outlined by Roberts et al (1976) to isolate the chloroplast and a haemocytometer to count them).

The results were analysed statistically using analysis of variance carried out on computer by means of Genstat IV (copyright 1984 Lawes Agricultural Trust (Rothamsted Experimental Station)) but no significant inter-clonal differences emerged.

Scoring System for in vitro Experiments

The microplantlets were cultured individually on 20 ml M & S shoot proliferation medium in 60 ml Steriline pots. The nature of these experiments did not facilitate accurate quantitative measurements to be taken without loss or disruption of the sterile environment around the microplantlets. Since the parameters were to be assessed repeatedly over the experimental period a scoring system was devised which permitted both the grading of observations and preservation of the micro-environment. This system is outlined below:

Growth score:-

0 = dead

1 = alive, little or no growth

2 = slight growth, some proliferation

3 = moderate growth and proliferation

4 = good growth and proliferation

5 = very good vigorous growth and proliferation

Despite the varying intensity with which a plant can be affected by chlorosis and production of anthocyanin, these were scored on a presence or absence basis only:

Chlorosis/Anthocyanin production score:

0 = absent

1 = present

The results obtained were statistically analysed using the Genstat IV computer programme. The X^2 test was performed on the chlorosis and anthocyanin data, while analysis of variance was carried out for the growth scores.

Comparison of Media

A comparison was made between the media proposed by Boxus (1974) for meristem initiation and subsequent shoot proliferation and those currently in use at the School of Agriculture for commercial strawberry micropropagation, ie 34.71 g/l Murashige and Skoog plant salt minimal organic mixture without agar (Flow laboratories catalogue number 26-000-22), IBA (indole-butyric acid) and BAP (benzyl-amino-purine) at 1 mg/l, NaFeEDTA at 40 mg/l, and 6 g/l Difco Bactoagar (0.6%) referred to as M & S, to determine which were best suited to the intended experiments.

Meristems were dissected from runners of the seven Cambridge Favourite clones received from Brogdale EHS and placed on either Boxus or M & S meristem initiation medium. After five weeks the explants were transferred to the corresponding proliferation medium and maintained at 22°C with 1-2 klx of light for 16h per day.

There were no apparent differences between meristem growth and performance on the two media at the initiation stage. However, differences became obvious upon transfer to the proliferation media when the M & S medium produced clusters of proliferating shoots with unfoliate leaves, while the Boxus medium gave rise to extended shoots with trifoliate leaves accompanied by a marked increase in callus formation, the few axillary buds formed being small and red.

Pennel (1987) experienced a similar lack of proliferation with Boxus shoot proliferation medium, while the size and colour of axillary buds was noted by Swartz et al (1981) and overcome by the addition of 1650 mg/l NH_4NO_3 to the medium. Boxus (1974), however, found that the presence of BAP (benzyl amino purine) in his medium encouraged the basal axillary buds to develop and grow quickly to produce new buds in turn, eventually giving rise to clusters of small proliferating shoots. The Boxus and M & S proliferation media contained identical concentrations of BAP, 1 mg/l, however, the different growth characteristics might be attributable to the lower level of BAP, 0.1 mg/l, present in the Boxus meristem initiation medium, whereas its M & S counterpart contains 1 mg/l.

It was decided to use M & S media for all future experiments as the clusters of shoots produced were more readily manipulated than their Boxus equivalents.

Induction of June Yellows in vitro

Braak (1955) managed to induce symptoms of June Yellows in apparently normal plants of the varieties Auchincruive Climax and Blakemore by growing them at 5°C with supplementary lighting of 7 klx for a minimum of 12 hours per day. The aim of the following experiment was to see whether June Yellows symptoms could be produced in plants growing in vitro, and also to determine the temperature regime most conducive to their appearance.

Proliferating microplants of Cambridge Favourite clones 1A, 3A, 3B, 3C, 3D, 8A, 8B, Y, FY and YM, previously grown under standard conditions of 22°C with

illumination of 1-2 klx for 16h per day, were cultured in 60 ml Steriline pots. Three temperature regimes were investigated; 4°C, 13°C and 22°C (control), day length and illumination intensity were as before. Each regime contained ten replicates of each clone where this was possible. The cultures were allowed to acclimatise for one week at the different temperatures, and four weeks later were graded according to growth, chlorosis and anthocyanin as previously outlined, and were then scored on a monthly basis. It should be mentioned that only for those plants exhibiting characteristic June Yellows symptoms, ie well defined chlorotic patches or mottle, was chlorosis scored as present. If chlorosis appeared to be due to the general ill-health of the plant it was scored as absent.

RESULTS AND DISCUSSION

This experiment ran for three months, during which time certain trends developed. From the start the known "yellows" susceptible clones at the two lower temperatures showed a significantly greater number of June Yellows-like chloroses than the "green" clones (see Table 1).

This differentiation was maintained throughout, with a far lower overall number of chloroses occurring at 22°C, where separation of "yellow" clones from "green" clones was less definite due to the lower number of chloroses within the "yellow" clones. Symptoms first became apparent in the plantlets held at 4°C, but once June Yellows-type chloroses began to appear in the 13°C material there was little difference between the number, or degree of severity, of chloroses produced at the two temperatures. However, the 13°C material is preferable to work with because of the temperature-enhanced growth rate which causes a faster rate of shoot proliferation and promotes leaf expansion at an earlier stage. Expanded leaves provide a more obvious display of June Yellows symptoms, and thus permit greater accuracy when scoring.

By the end of the experiment the "yellow" clones had achieved nearly 100% positive chlorosis scores both at 4°C and 13°C and a low percentage of "green" clones held at 4°C and 13°C were also exhibiting some chlorosis. The onset of chlorosis in the "green" clones is unlikely to have been due to nutrient deficiencies, vitrification or senescence incurred after three months in Steriline pots as this trend was not observed in the 22°C material which achieved the fastest growth rates and consequently could be expected to show symptoms of media exhaustion or senescence first. Differences between the 4°C and 13°C material were more marked for growth and anthocyanin production.

Incidence and intensity of anthocyanin production decreased with increasing temperature (see Table 2). Production was most active at 4°C when the pink pigmentation could be dense enough to mask the natural green or yellow colour of the plantlet, which made scoring for chlorosis difficult. In addition, at 22°C the rate of growth of the plantlets was such that large portions become shielded from view by overlying layers of shoots. Consequently, if anthocyanin was not visible on the outside of the proliferating cluster it was recorded as absent although it might have been present on the internal shoots. The observed differences between clones were minimal and none were maintained. A greater anthocyanin incidence was noted towards the end of the experimental period in the "yellow" clones held at 22°C although there is currently no explanation for this.

The initial mean growth scores for the 4°C and 13°C experiments were very similar, but the maximum growth rate occurred at 22°C which is the optimum temperature for the micropropagation of strawberries. The gap between the 4°C and 13°C sets widened with time. Initially the "yellow" clones held at 4°C showed the lowest growth rates though these were not statistically significant. However, during the mid and later stages FY and YM grew with increased vigour to finish amongst the clones with the overall highest growth scores, ie 3D, 1A and 8A. FY and YM did not suffer from early stunting at the higher temperatures. Clone Y achieved the least

growth at all temperatures and remained retarded throughout the experiment; it is the most difficult of the clones to maintain in culture because it tends to form callus (some portions of which die off) and few shoots. This suggests that the differences between Y and FY & YM in growth habits are greater phenotypically, and possibly genotypically, than those between FY and YM and the "green" clones. Generally the stunting associated with June Yellows in vivo appears to be short-lived in vitro.

Experiment II

In order to investigate whether plantlets held at low temperatures for reasonably long periods would produce June Yellows symptoms if the temperature was increased, thus mimicking the onset of spring conditions in the field, the material from the first temperature experiment was used to set up the following.

All replicates held at 4°C and 13°C were split so that two new cultures were formed from each. One set of material remained at the original temperature, 4°C or 13°C, while their genetically identical sets were moved up to 22°C. The cultures acclimatised for one week and then were scored on a fortnightly basis, for the same criteria as before, over a period of two months.

13°-13°C (control) and 13°-22°C Sets of Material

A clear distinction was maintained throughout the experiment between "green" and "yellow" clones (as shown in Table 3), with the latter being responsible for nearly all instances of chlorosis. No statistically significant difference concerning the number of chloroses evident in the two 13°C treatments became obvious during the two month run of the experiment. Initially there was a slight tendency for the material moved up to 22°C to produce new chloroses, but this was minimal and was not maintained; so towards the end the 13°C control exhibited marginally higher levels of chlorosis. Very few cases of June Yellows-type chlorosis appeared amongst the "green" clones in either set and those expressed at 22°C disappeared again quite quickly as the resumption of normal leaf colouration, and hence loss of symptoms, is

promoted under these conditions.

4°-4°C (control) and 4°-22°C Sets of Material

The 4°C control and 4°-22°C sets of plantlets were not differentiable statistically for chlorosis scores until 7 weeks into the experiment. A steady increase in the occurrence of June Yellows-type chloroses was observed for the first month after sub-culturing in almost all the clones in both 4°C treatments, although clone Y tended to lag behind its fellow "yellow" clones FY and YM (which maintained close to 100% for chlorosis throughout), and finally attained a chlorosis level more akin to that of the "green" clones. The performance of Y may be due to a number of features more common to it than to the other clones, ie masking of true colour by high anthocyanin levels (particularly in this 4°C or ex 4°C material), death, or display of a type of chlorosis more indicative of ill-health which would disqualify plantlets from being counted for this parameter.

In the 4°C control material the number of plants of "green" clones exhibiting symptoms increased rapidly (Figs 1-4) resulting in scores as high as 8/10 being recorded for 3A and 3B), although the average number of affected plants for "green" clones was nearer to 6/10. By the end of the experiment the number of "green" clone chloroses had declined slightly in some clones, while it continued to increase in others. The maximum level of chloroses in the 4°-22°C material was achieved by the end of the first month, thereafter a slow decline ensued as affected plants lost their symptoms and regained their original non-chlorotic appearance.

These results show that more June Yellows-type chloroses were produced and/or induced at 4°C over a period of about 6 months than were revealed at higher temperatures, even in material preconditioned at 4°C for 3 months. The implication is that the division between "yellow" and "green" clones, ie that "yellow" clones are susceptible to, or have, the disorder while "green" clones are resistant or free from it, is not as clear cut as was originally hoped. All the clones used in this experiment

developed June Yellows symptoms sooner or later, to a greater or lesser degree. It must be admitted that the "yellow" clones produced symptoms more readily and in greater numbers throughout that the "green" clones. However, it would seem that given an appropriate stimulus, ie periods of 5-6 months at 4°C, supposedly "green" clones can be forced to produce June Yellows-type chloroses, in such quantities that some clones would rank almost on a par with known "yellows" clones. Consequently a profile of susceptibility has been revealed, which indicates that all clones tested have the potential to develop the disorder, but that some are more prone to it than others.

The rate of symptom development is related to the length of time spent at low temperatures, and it is possible that this treatment causes some physiological change in the plants by means of the production, or inhibition, of a trigger metabolite or plasmagene (Wills, 1962) which, once it exceeds or falls below a certain threshold level, results in the expression of symptoms. Therefore, the apparent susceptibility profile would be directly related to the actual threshold level prevailing in each clone or the production rate of the trigger substance. Thus the observed interval between the release of a cultivar on the market and the first indications that it has June Yellows could be attributed to a gradual, and possibly reversible, build up of this trigger metabolite or plasmagene, as a result of periodic or continuous exposure to the stimulus so that several years may elapse before the threshold level is exceeded. The threshold level, or rate of production of the trigger, may vary between genotypes, thereby accounting for the inter-varietal and inter-clonal differences observed in rate of symptom expression. In addition, if higher temperatures broke down or restarted production of the trigger substance, or inhibited the activity of the plasmagene, it could result in the threshold being crossed in the opposite direction, in which case a slow loss of visible symptoms might be expected, which related to field observation (Plakidas, 1932).

The remission of symptoms observed in both the 4°C and 13°C material when moved to a 22°C environment fits well with Braak's (1955) report that plants which had exhibited symptoms when held at 5°C experienced a greening of the leaves within 2-3 weeks at 20°C, and a full recovery within a month. The lag observed in some of our material may be due to the extended period of exposure to low temperatures prior to being moved; Braak made no mention of the length of 5°C treatment to which these plants were subjected. Affected "green" clones tend to return to a normal appearance more readily than "yellow" clones upon an appropriate change of conditions. This may be because they have not exceeded the proposed threshold point to the same extent as the more sensitive "yellow" plants in the same treatment, although the metabolic reversal might occur at the same rate in both clone types.

Braak observed no further increase in chlorosis in plants held at 5°C after 10 weeks, and also asserted that "normal", ie "green" clones remained green at both 5°C and 10°C, however, results presented here indicate that a period of 10 weeks is not long enough to determine whether a plant in an in vitro situation has the ability to produce symptoms. Our results also show that "normal" clones go yellow most rapidly at temperatures close to 5°C and 10°C, and that temperatures close to 22°C are required before a steady absence of symptoms can be achieved.

Anthocyanin

Scoring for anthocyanin production revealed no obvious divisions between "yellow" and "green" clones, the only significant statistical differences occurring between clones lying at the extremities. However, there was considerable variation between the different temperature treatments, which reinforced the temperature-related nature of anthocyanin production (see Table 4). The control sets maintained steady scores which increased slowly with time; the 4°C control set gained significantly higher scores throughout. The sets elevated to 22°C immediately showed reduced levels of anthocyanin in comparison to their controls and by the end of 8 weeks they achieved the lowest scores and were markedly different from their controls.

Anthocyanin production is related to maintenance temperature, and is generally believed to be a stress reaction. June Yellows might also be a reaction to stress, however, the lack of correlation for chlorosis with anthocyanin production between affected and unaffected clones rules out a connection between the two responses to the common stimulus of low temperature.

Growth Scores

Initially the 4°-22°C and 13°-22°C sets closely resembled their respective controls with the 4°C sets having significantly lower scores than the 13°C sets. A variety of regroupings were observed until after 9 weeks each set was shown to be statistically different at the 5% level to all of the others, with the final ranking, from lowest to highest, being as follows: 4°C control, 13°C control, 4°-22°C, 13°-22°C.

It is interesting to note that although manifestation of June Yellows symptoms in the field is always accompanied by stunting of the plant this does not necessarily follow in vitro where the individual genotypes have a greater opportunity for expression, possibly because they are unaffected by other stress conditions prevalent in vivo. Clone Y achieved significantly lower scores at all temperature options throughout, the sole exception being its performance at the 9 week stage at 4°C, when it revived briefly. This was the only clone to show any consistently significant inter-clonal differences. Despite Y's poor performance, the other "yellow" clones FY and YM maintained a higher growth rate and ranked with the most vigorous of the "green" clones almost without exception, although they did grow slowly at first, and it is possible that this initial retardation cannot be overcome under field conditions.

CONCLUSIONS

These results show that expression of June Yellows-like symptoms can be induced in vitro. All clones tested showed a greater or lesser degree of susceptibility to the disorder when given appropriate temperature treatments. Time consuming tests could be run to assess comparative clonal or varietal resistance to June Yellows but it seems

unlikely that clones from affected varieties would be totally unsusceptible.

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TABLE 1. Induction of June Yellows in vitro: Chlorosis Scores

Clones/Months from start		4°C			13°C			22°C	
	1	2	3	1	2	3	1	2	3
Yellow[1] with symptoms	19	21	29	19	28	29	18	16	9
Yellow[1] without symptoms	11	9	1	10	1	0	12	14	21
Green[2] with symptoms	17	5	7	1	0	6	0	0	0
Green[2] without symptoms	48	60	58	65	66	60	66	66	66

1 ie yellows susceptible clones, Y, FY and YM, with a maximum of 30 and minimum of 29 plants.

2 ie clones supposedly unsusceptible to June Yellows although from a Yellows-affected cultivar. Clones 1A, 3A, 3B, 3C, 3D, 8A and 8B, with a maximum of 66 and minimum of 65 plants.

TABLE 2. Induction of June Yellows *in vitro*: Anthocyanin Scores

Clones/Months from start		4°C 1	4°C 2	4°C 3	13°C 1	13°C 2	13°C 3	22°C 1	22°C 2	22°C 3
Yellow[3]	with anthocyanin	22	30	30	6	29	29	7	19	24[5]
	without anthocyanin	8	0	0	23	0	0	23	11	6
Green[4]	with anthocyanin	44	65	65	21	64	66	5	39	50
	without anthocyanin	41	0	0	45	2	0	61	27	16

3 As in Table 1.

4 As in Table 1.

5 Colour much less intense than at lower temperatures.

TABLE 3. Experiment II: Chlorosis Scores

Clones/Weeks from start		4°-4°C				4°-22°C				13°-13°C				13°-22°C			
		3	5	7	9	3	5	7	9	3	5	7	9	3	5	7	9
Yellow[6]	with symptoms	24	23	24	23	18	26	25	26	20	23	23	24	22	23	26	23
	without symptoms	6	7	6	7	12	4	4	3	9	6	6	3	7	6	3	5
Green[7]	with symptoms	4	20	40	43	8	18	12	9	0	0	1	1	1	2	0	0
	without symptoms	61	45	25	22	57	47	52	55	66	66	65	65	65	64	64	64

6 As in Table 1; maximum of 30, minimum of 27 plants.

7 As in Table 1; maximum of 66, minimum of 64 plants.

TABLE 4. Experiment II: Anthocyanin Scores

Clones/Weeks from start		4°-4°C				4°-22°C				13°-13°C				13°-22°C			
		3	5	7	9	3	5	7	9	3	5	7	9	3	5	7	9
Yellow[8]	with anthocyanin	29	29	29	29	28	23	6	3	17	18	14	18	13	5	2	4
	without anthocyanin	1	1	1	1	2	7	23	26	12	11	15	9	16	24	27	24
Green[9]	with anthocyanin	63	63	61	65	55	51	15	15	66	62	57	61	57	43	28	28
	without symptoms	2	2	4	0	10	14	49	49	0	4	9	5	9	22	36	36

8 As in Table 1.

9 As in Table 1.

Figs. 1-4. Comparison of number of plantlets per clone displaying June Yellow-like chloroses (maximum of 10) 3, 5, 7 and 9 weeks after splitting

Fig 1. 3 weeks after splitting

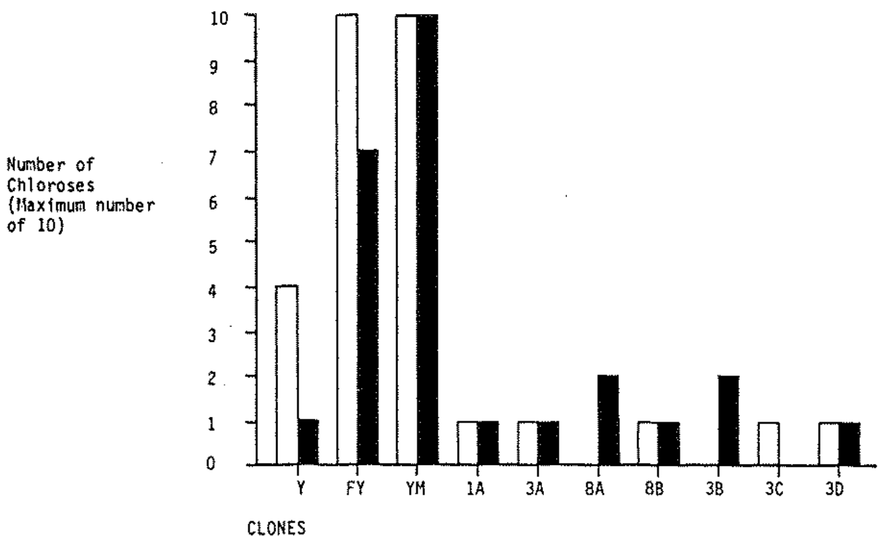


Fig 2. 5 weeks after splitting

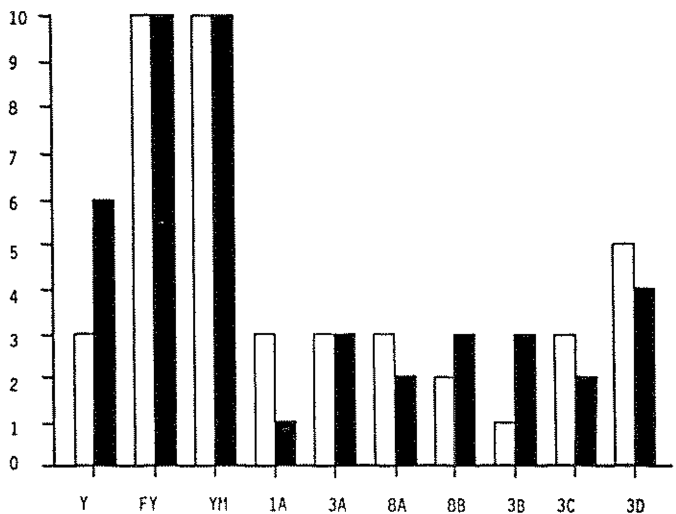


Fig 3. 7 weeks after splitting

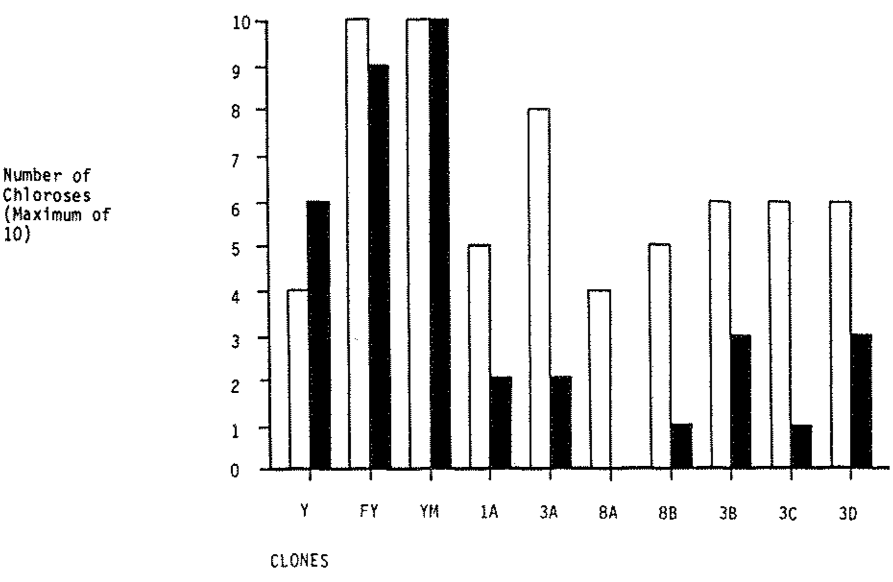
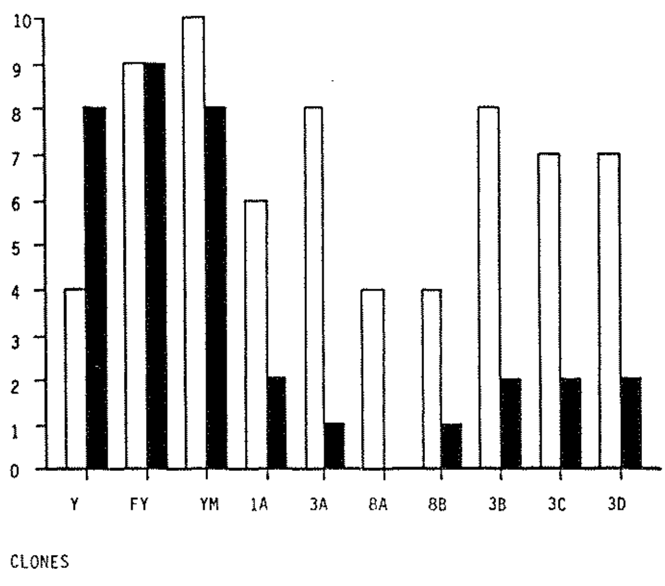


Fig 4. 9 weeks after splitting



THE EFFECT OF ALTERNATING TEMPERATURE ON *IN VITRO* PRODUCTION OF JUNE YELLOWS OF STRAWBERRY (*FRAGARIA ANANASSA* DUCH.)

by

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ABSTRACT

June Yellows affected clones of cultivar Cambridge Favourite, and unaffected clones of cultivars C. Favourite, Auchincruive Climax and Bogota were grown in vitro and subjected to four warm day (12 h or 16 h at 22°C or 13°C)/cold night (12 h or 16 h at 4°C) treatments for four months. In both 13°C/4°C treatments and the 22°/4°C 16 h/18 h treatment only those clones previously affected produced symptoms. The 22°C/4°C 12 h/12 h treatment also produced a low level of chloroses in previously unaffected clones which later showed remission of symptoms. Reasons behind these observations are discussed.

INTRODUCTION

The unpredictable nature of June Yellows has caused vast problems for growers and breeders in the modern-day strawberry industry. In the absence of an established pathogenic causal agent (Plakidas, 1932; Guba, 1932, 1933) or sensitivity to a nutrient deficiency (Morris and Afanasiev, 1944) it would seem that it is probably genetic in origin. The condition is heritable (Anderson, 1935), but inheritance studies show that it is probably not controlled by the nucleus (Maas, 1984) ; thus a rogue plasmagene, (Williams, 1955) closely adapted virus (Jones et al, 1986; Wills, 1961, 1963), mycoplasma-like organism (Huhtanen, 1971) or other cytoplasmic factor may be responsible.

There are severe economic repercussions for any new variety found to be susceptible to the disorder. Breeding non-June Yellows-susceptible stock is an unenviable task with massive risks, especially as there can be a lag period of up to 10-30 years between the release of a cultivar and the first appearance of symptoms (Braak, 1955). In addition, there is no way of testing previously symptom-free parental plants from susceptible varieties to establish whether or not they have the potential to develop June Yellows at a later date.

The symptomology of the condition has been described in many papers, most recently by Hughes (1989). Briefly, symptoms include a wide, but distinctive, range of chlorotic mottles, flecks and streaks on the leaves. These appear in the spring, mainly towards the end of April until the middle of May. During the summer months a certain degree of remission may occur (Plakidas, 1932), the extent of which depends upon the original severity of symptoms. Symptoms may reappear in the autumn in a more mild form. Yield can be reduced by more than 50% (Wilson, 1955) and seriously affected plants suffer from stunted growth (Demaree & Darrow, 1937).

The simultaneous onset of symptoms across sizeable geographic areas (Braak, 1955) suggests that the condition is environmentally sensitive and that symptom expression is a reaction to a specific combination, or band, of climatic circumstances. Williams (1955) attributes this to a progressive change in clones that may or may not be environmentally-linked which results in simultaneous mutations causing green plants to exhibit yellows.

The propensity of strawberry plants to succumb to viral infections has caused problems for growers wishing to obtain clean stock for planting. Although virus-free plants can be produced it is not yet possible to multiply them up at a commercial rate except by micropropagation. The inherent danger of using this technique is the proliferation of any genetic faults present in the parent to all daughter plants. Consequently, stringent testing of parental material for suitability is recommended, although, to date, there is no means of testing for the proclivity of plants to June Yellows. The following experiment was part of a series (Wade, 1990 submitted) performed in an effort to develop such a test to enable potential micropropagation material to be screened for the disorder.

ALTERNATING TEMPERATURES

Materials

Seven NSA clones of strawberry cultivar Cambridge Favourite, 1A, 3A, 3B, 3C, 3D, 8A, and 8B, which had never exhibited June Yellows symptoms, were provided by Brogdale EHS as pot-grown plants. (Clone 8A has since been eliminated from NSA mother stocks at East Malling by the NFT Strawberry Panel after developing transient yellows (Posnette, 1955) but is maintained at the East of Scotland College of Agriculture (ESCA) for research purposes). "Yellow" Cambridge Favourite clones Y and YM were provided as runners from plants known to be suffering from the disorder during a multi-centre trial run by ESCA at Castle Huntly, while another Cambridge Favourite "Yellow" clone, FY, originated from the ESCA field trials in 1987. Plants of the variety Bogota were supplied by Mr D Mitchell from the Bush Estate, East Lothian, and plants believed to be Auchincruive Climax (official descriptor unavailable) were donated from Mr D Reid's private collection.

In vitro cultures were initiated from meristems (Wade, 1990 submitted) and maintained in glass jars on Murashige and Skoog shoot proliferation medium, ie 34.71 g Musharige and Skoog plant salt minimal organic mixture without agar (Flow Laboratories catalogue number 26-000-22), IBA (indole-butyric acid) and BAP (benzyl-amino-purine) at 1 mg/l, NAFe EDTA at 40 mg/l and 6 g/l Difco Bactoagar (0.6%), at 22°C with 16 h of 1-2 klx light per day. The proliferating microplants used in experiments were cultured in 60 ml steriline pots on 20 ml M&S shoot proliferation medium.

Methods

June Yellows is not evident in areas close to the equator, but the reasons for this are not known. Annual charges in daylength and the alternation of high and low temperatures, which accompany day and night at certain times of the year in non-equatorial regions, constitute the most obvious departure from climatic conditions at

the equator. In vivo June Yellows symptoms peak at a time when the day length is changing rapidly and there is a marked difference between day and night temperatures; the following experiment was set up to establish the extent to which these conditions affect the occurrence of June Yellows symptoms in vitro.

Four temperature/light combinations were investigated:

Treatment A = 13°C light/4°C dark
12 h 12 h

Treatment B = 13°C light/4°C dark
16 h 8 h

Treatment C = 22°C light/4°C dark
12 h 12 h

Treatment D = 22°C light/4°C dark
16 h 8 h

Cambridge Favourite clones Y, FY, YM, 1A, 3A, 3B, 3C, 3D, 8A and 8B, Auchincruive Climax (AC) and Bogota (BOG) were cultured in Steriline pots and allowed a week to stabilise at 22°C with 16 h 1-2 klx light per day after which 10 replicates of each clone were placed in each temperature/light regime and scored a week later. Subsequent scorings were taken 2, 4, 6, 8, 10, 12, 14 and 16 weeks after entry to the system. The microplants were graded for growth, chlorosis and anthocyarin production using the system outlined below which permitted repeated assessments and avoided loss of or disruption to the sterile environment.

Growth score:-

0 = dead

1 = little or no growth

2 = slight growth, some proliferation

3 = moderate growth and proliferation

4 = good growth and proliferation

5 = very good vigorous growth and proliferation.

Chlorosis/Anthocyanin Production

These parameters were scored on a presence or absence basis only although the plants exhibited a wide range of intensities for both. Chlorosis was scored as present only for those plants which exhibited characteristic June Yellows symptoms with well defined chlorotic patches or mottle; if chlorosis appeared to be due to the general ill-health of the plant it was recorded as absent.

Present = 1

Absent = 0

The results obtained were statistically analysed by the Genstat IV computer program (copyright 1984 Lawes Agricultural Trust (Rothamsted Experimental Station)). The X^2 test was performed on the chlorosis and anthocyanin data, while analysis of variance was carried out on the growth scores. Any significance cited is significant at the 5% level.

RESULTS AND DISCUSSION

Chlorosis

All plantlets were symptom free when cultured, however, a significant separation of the "yellow" clones, Y, FY & YM which became chlorotic, from the symptomless "green" clones, 1A, 3A, 3B, 3C, 3D, 8A, 8B, AC, and BOG, was visible from week 4 onwards across all treatments except Treatment A in which this division became apparent from week 8 (see Table 1). Treatment A did not produce a directly comparable number of chloroses until week 10. This lag in symptom expression may be attributed to the observed delay in leaf expansion and subsequent revelation of symptoms experienced by these plants which probably resulted from being kept at the coolest overall combination of maintenance temperatures, ie 13 °C light/4 °C dark 12 h/12 h.

The combinations of lower temperatures in Treatments A and B, ie 13°C and 4°C, prevented June Yellows affected plants from losing their symptoms and regaining their original green colour. By comparison the higher overall combinations of temperatures used in Treatments C and D resulted in remission of symptoms in up to 30% of chlorotic plants (see Table 1, Treatment C at weeks 8 and 16). A high proportion of recovery occurred in the "green" clones in which symptoms were transitory. Recovery can be induced or promoted by exposure to a warm environment; Braak (1955) achieved remission in pot grown plants at temperatures between 10°C-20°C and we (Wade, 1990 submitted) observed it at 22°C in vitro.

Thus, the conditions provided by Treatment C were the most successful at inducing June Yellows symptoms in "green" plants to a low but significant level, and also at assisting recovery. These results seem to indicate that for induction of June Yellows symptoms in previously affected material, ie "yellow" clones, the overall mean temperature was the most important factor and that daylength only affected the rate of growth and expansion of leaves and not symptom expression. Hence Treatments A and B produced and maintained 100% chlorosis scores for the "yellow" clones in comparison to the approximate 80% maximum achieved in the same clones by Treatments C and D. However, for the "green" clones, which were previously unaffected, the overall difference in temperatures, coupled with the duration of the cold dark period seems to have exerted the major effect.

The 18°C temperature shift and longer cold period of Treatment C permitted the induction and expression of a low level of "green" clone chloroses which were not maintained under these conditions. The 16 h warm/light period of Treatment D possibly provided conditions that promote recovery to such an extent that June Yellows was not expressed although the disorder might have been induced or present in an inactive state. It is possible that a balance could be struck by retaining the temperature difference of Treatment C and reducing the length of the photoperiod to

enable a greater expression of "green" clone chloroses and simultaneously prevent remission of symptoms.

There was very little to distinguish between the effects of the equatorial and temperate daylengths, (12 h and 16 h respectively) which agrees with Braak's findings (1955) when he tested the response of whole pot-grown plants to the effect of a variety of photoperiods and, in one instance, an alternation of light and dark temperatures (8.5°C and 1.5°C) over a 10 week period. He concluded that neither the length of illumination, nor the fluctuation of temperature around a mean of 5°C exerted any effect on the proportion of June Yellows expressed. However, when treatments with the same day length were compared, the lack of similarity between the final results for Treatments A & C, and Treatments B & D implied that the known June Yellows susceptible plants were more sensitive to temperature than photoperiod, although it is undeniable that the two factors are closely linked in the field at temperate latitudes. This contradicts Braak's data, but it is not surprising given the difference in the temperatures tested and the duration of the experiment.

Clone YM scored significantly lower than the other "yellow" clones Y and FY until week 12. From week 12 onwards clone Y scored significantly more chloroses than FY and YM (see Table 2). There is currently no explanation for these differences.

Anthocyanin Production

The overall number of plants that produced anthocyanin increased throughout the experiment. Auchincruive Climax scored significantly higher than the other clones from weeks 1-4 after which the other clones' scores rose to similar levels. From week 8 until the end of the experiment clone Y achieved significantly lower scores than the other clones.

There were very few significant differences in anthocyanin production between the treatments and these were irregular and found briefly between the extremities. The temperature-related nature of anthocyanin production observed in earlier experiments

(Wade, 1990, submitted) was not obvious here, especially as all the treatments ended up with final scores between 105/120 and 113/120, for which the statistical difference is negligible.

Growth Scores

Statistical analysis of growth scores revealed no consistent significant differences between clones, clones within treatments or treatments until week 6 after which Treatment D maintained significantly greater scores. It seems that the range of temperatures used were not of a kind to produce statistically valid growth variations across the span of treatments.

CONCLUSIONS

The photoperiod has less effect on the induction of expression of June Yellows symptoms in vitro than does either the overall mean temperature or the size of the difference between alternating high and low maintenance temperatures.

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TABLE 1. Chlorosis Scores: To show overall distribution between treatments and clone types.

Weeks from start		1	2	4	6	7	10	12	14	16
Treatment A (13°C/4°C 12 h/12 h)	Yellow	0	0	2	3	10	25	30	30	30
	Green	0	0	0	0	0	0	0	0	0
Treatment B (13°C/4°C 16 h/8 h)	Yellow	0	1	11	15	26	29	29	30	30
	Green	0	0	0	0	0	0	0	0	0
Treatment C (22°C/4°C 12 h/12 h)	Yellow	0	2	13	16	25	20	23	23	20
	Green	0	0	1	2	7	1	4	0	0
Treatment D (22°C/4°C 16 h/18 h)	Yellow	0	4	16	18	22	22	18	19	16
	Green	0	0	1	0	0	0	0	0	1
	Total	0	7	44	54	90	97	104	102	97

"Yellow" clones scored out of 30; "Green" clones scored out of 90

TABLE 2. Chlorosis Scores: To show distribution of chlorosis amongst the known yellows affected clones.

Weeks from start		1	2	4	6	7	10	12	14	16
Treatment A (13°C/4°C 12 h/12 h)	Y	0	0	0	0	3	9	10	10	10
	FY	0	0	1	2	5	9	10	10	10
	YM	0	0	1	1	2	7	10	10	10
Treatment B (13°C/4°C 16 h/8 h)	Y	0	0	1	4	9	10	10	10	10
	FY	0	1	6	8	10	10	10	10	10
	YM	0	0	4	3	7	9	9	10	10
Treatment C (22°C/4°C 12 h/12 h)	Y	0	2	7	6	10	10	9	10	9
	FY	0	0	5	7	8	5	10	7	7
	YM	0	0	1	3	7	5	4	6	4
Treatment D (22°C/4°C 16 h/8 h)	Y	0	2	9	10	10	10	10	10	10
	FY	0	2	6	6	9	9	6	6	5
	YM	0	0	1	2	3	3	2	3	1

Scores are out of 30

Part 2

Field Distribution Studies

Two new sites were investigated this year but no distinct pattern of distribution was seen. The sites were inspected again after the harvest, however, at this time the re-emergence of June Yellows symptoms could not be distinguished from those of senescence. These were both first year plantations which it might be worth continuing to monitor next spring and possibly the year after.

Effect of Cold Shock

In vitro Cambridge Favourite clones 3A, June Yellows affected YM, and plants of Auchincruive Climax (as provided by Mr Reid, but not cross referenced to the original variety descriptor, which could not be obtained) were exposed to a cold shock of 4°C for 30 mins, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h or 12 h then maintained at 22°C with 1-2 kl for 16 l/day, in order to discover whether symptoms could be induced by a sudden drop in temperature. There were virtually no symptoms of June Yellows produced as a result of this treatment. YM showed a maximum of 3/10 affected plants in one treatment but, as YM plants can show symptoms when maintained at 22°C, it seems unlikely that these were induced by the cold shock. It seems that low temperature exposures greater than 12 h in duration are required before June Yellows is induced.

Experiments with DAPI

4'-6-diamidino-2-phenylindole (DAPI) is a DNA-binding fluorochrome which has been used to show the presence of mycoplasma-like-organisms (MLO's) in fresh and fixed tissue by means of a rapid compression technique. Yellows-free and known-yellows material stained with 1% DAPI was examined by fluorescence microscopy. The fluorescence from nuclei, chloroplasts, and possibly mitochondria, was convincing, however no MLO's could be identified even when fluoroscene cross-staining was used to highlight the vascular tissue (in which MLO's are most frequently located). Similar tests were performed on both healthy and MLO-infected potato

tissue, although none were observed in either fresh or fixed material. The slides produced by these methods lacked the clarity required to distinguish MLO's from background or underlying fluorescence.

Effect of Pre-heating

Stock cultures of Cambridge Favourite clones 3A, 3B, 8A, Bogota and yellows-affected C. Fav. FY and YM, were maintained at 28°C 1-2 kl x 12 h/day for 9 weeks. These were then cultured into 60 ml steriline pots on shoot proliferation medium and given 12 days to stabilise at 22°C before the temperature was gradually reduced to 5°C over a period of 7 days. A control was provided with plantlets that had previously been kept under standard conditions at 22°C.

The pre-heated plants showed very high rates of nitrification and growth abnormalities, very few appeared healthy, and none produced classic June Yellows symptoms. Consequently, no valid comparison could be made between the treatments and the experiment was dismantled after 7 weeks.

Antibiotic Experiment

Phytotoxicity tests on standard shoot proliferation medium containing 1 mg, 10 mg or 100 mg/l ribavirin (an antiviral agent) have indicated that 10 mg/l is the highest of the concentrations tested that gives an acceptable plantlet survival rate. An experiment has been set up using Cambridge Favourite clones FY, YM, (both yellows affected) 3A and 3D, and clones of Auchincruive Climax, some of which had been chilled between 4-10°C for several months previously, on normal shoot proliferation medium and shoot proliferation medium containing 10 mg/l ribavirin. The cultures are being kept at 4°C to maximise symptom expression. There are no results available at present, but these will emerge over the following months.