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Determining the Optimal Timing of Fruit Harvest in Transgenic Tomato Expressing F1-V, a Candidate Subunit Vaccine against Plague

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Abstract. **Changes in the amounts of F1-V, an antigen fusion protein and a candidate subunit vaccine against plague, and total soluble protein (TSP) in green fruit of transgenic tomato plants were investigated to identify the optimum harvest timing to maximize the F1-V yields. Two T_2 progenies of the transgenic plant, '22.11.21' and '22.11.5', were grown. The F1-V concentration rapidly decreased at the beginning of the green stage and decreased to less than 5% of the initial concentration at the late green stage in '22.11.21'. The F1-V concentration also decreased as fruit size increased in '22.11.5', but the pattern of the decrease was linear and different from that in '22.11.21'. The concentration of TSP also decreased with fruit growing in both plants. When calculated on a whole fruit basis, the F1-V content linearly decreased with increasing fruit size in '22.11.21'. In '22.11.5', the F1-V content per fruit also tended to decrease from the middle to late green stage. Based on these observations, collecting small green fruits without pruning was proposed as a harvest practice that may maximize the F1-V yields. Thus, the optimum protocols for harvesting and pruning for plant-made pharmaceutical production may be substantially different from those currently used in commercial hydroponic greenhouses for fresh market tomato.**

Plant-made pharmaceutical (PMP) production is attracting considerable interest. The PMPs include antigens, hormones, growth factors, blood proteins, cytokines, enzymes, and antibodies; and both stable transformation and transient expression with plant virus vectors is used as plant-based expression systems. Such PMP production systems have several advantages, including lower cost, higher scalability, few downstream manufacturing processes, and lower contamination risk of potential human pathogens or toxins compared with traditional fermentation- or bioreactor-based systems using recombinant microbes or transformed animal or mammalian cells (Daniell et al., 2001; Mason and Arntzen, 1995; Mason et al., 2002; Thanavala et al., 2006; Twyman et al., 2003). Greenhouse tomato (*Solanum lycopersicum*) production is considered to be one of the most suitable production systems using transgenic plants for such high-value proteins in terms of relatively efficient transformation system available (Mason et al., 2002), high biomass yield (Twyman et al., 2003), containment to prevent transgene flow to the outside (Twyman et al., 2003), and environmental control for steering the plant growth to maximize the protein productivity with minimum input of available resources (Matsuda et al., 2009).

The expression level of target protein per unit of harvested biomass is one of the determinants for cost-effectiveness of the system (Twyman et al., 2003). A high protein concentration is also crucial if it is the intent to produce an orally delivered vaccine after minimal processing of plant material (e.g., freeze-drying), which is immunogenic in a reasonably small dose (Alvarez et al., 2006, 2008; Mason and Arntzen, 1995; Mason et al., 2002).

To increase the expression level of a transgene, various genetic and molecular techniques have been shown to be effective, including use of 5′ translational enhancers (Mason et al., 1998), synthetic genes with plant-favored codons (Alvarez et al., 2006; Mason et al., 1998), alternative polyadenylation signals (Richter et al., 2000), specific subcellular targeting signals (Alvarez et al., 2010; Richter et al., 2000), and suppression of gene silencing (Alvarez et al., 2008).

The recombinant protein concentration in any plant tissue is determined not only genetically, but also physiologically and environmentally. Antibody or antigen protein concentration in leaves of transgenic tobacco plants decreases during leaf development and increases at low temperature combined with high light intensity (Stevens et al., 2000) and increases under moderate water stress (Stevens et al., 2007). Less attention has been paid to the effect of physiological and environmental factors on the concentration and yield of target proteins than the genetic aspects. Developing fruit generally go through dynamic changes in metabolites and transcript levels as recently demonstrated in tomato (Carrari et al., 2006). The dynamics of target protein content needs to be quantified to optimize protein production in developing transgenic tomato fruit.

Alvarez et al. (2006) generated transgenic tomato plants with the *Yersinia pestis f1-v* fusion gene encoding for F1-V, an antigen fusion protein. Freeze-dried powder of the fruits is an orally delivered vaccine candidate against plague. The immunogenicity of F1-V against a challenge with subcutaneous *Y. pestis* was confirmed in mice that had been vaccinated orally with the freeze-dried fruits (Alvarez and Cardineau, 2010). We recently characterized the F1-V protein productivity as well as the growth and development of the F1-V transgenic tomato plants grown in a temperature-controlled greenhouse with cultural practices adopted by commercial hydroponic tomato growers (Matsuda et al., 2009). However, the fruit F1-V concentrations obtained under greenhouse conditions were low, only 16% of those previously observed in a growth chamber (Alvarez et al., 2006). The differences in concentration suggest significant influences of growing conditions and/or harvesting practices. Fruit F1-V markedly decreased during fruit ripening from the green stage to the breakers stage (Alvarez et al., 2006). The possible degradation of F1-V during the rapidly growing green fruit stage has not been examined.

In this study, we investigated how the concentrations and contents of F1-V and total soluble protein (TSP) varied with the stage of green fruit development in the transgenic tomato plants. Our goal was to identify

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the optimum harvest timing within the green stage to maximize the F1-V yields. Green fruits of various sizes were harvested at one time for each of two T_2 progenies of the *f1-v* transgenic plants, '22.11.21' and '22.11.5', and the relationship between the amount of F1-V or TSP and fruit size was analyzed.

Materials and Methods

Plant material. The primary transformant (T_0) of the transgenic tomato plants expressing F1-V protein had been obtained previously by *Agrobacterium*-mediated transformation of a wild-type 'TA234' with the *Y. pestis f1-v* fusion gene (Alvarez et al., 2006). Expression of the *f1-v* fusion gene was driven by the CaMV 35S promoter. One plant of its T_1 progeny, '22.11', showed a high F1-V expression level of 11% of fruit TSP (Alvarez et al., 2006). The '22.11' selection was allowed to self-fertilize, and two T_2 progenies, '22.11.21' and '22.11.5', were used in this study. These two T_2 progenies were selected based on their relatively high F1-V expression levels and on their visual normality. We observed abnormal morphology and physiology in several other T_2 plants such as excessive proliferation of small flower buds within a truss; downhanging, curly, distorted leaves; and/or magnesium deficiency-like symptoms of chlorosis and necrosis on leaves (Matsuda et al., 2009). For '22.11.5', three rooted cuttings were used in the experiment.

Growth conditions and experimental setup. The '22.11.21' plant was grown hydroponically with a high-wire system in an acrylic greenhouse (BSL-2) in Tucson, AZ, as described in detail in Matsuda et al. (2009). The seed (T_2) was sown on 15 Aug. 2007 and was transplanted onto a rockwool slab on 20 Sept. 2007. Mean, maximum, and minimum daytime air temperatures, determined during sample fruit growth from 17 Jan. 2007 to 13 Mar. 2008, were ≈21, 24, and 17 °C, respectively. Mean, maximum, and minimum nighttime temperatures during this period were ≈18, 20, and 17 °C, respectively. Daily mean relative humidity was maintained above 60%. Daily photosynthetic photon flux (*PPF*) integral on sunny days was between 15 and 25 $mol \cdot m^{-2} \cdot d^{-1}$.

The cuttings of '22.11.5' (T_2) with three to five leaves were rooted in a growth chamber. After rooting, the plantlets were transplanted to 10 × 10-cm rockwool blocks (Grodan BV, Roermond, The Netherlands) between 23 June and 8 July 2008, acclimatized for 1 week, and grown in the same greenhouse as used for '22.11.21' until Oct. 2008. The plants were subirrigated once a day with a half-strength modified Hogland nutrient solution, in which the composition was described subsequently. On 5 Aug. 2008, each of the three uniform plants with ≈50-cm plant height and 12 to 13 true leaves was transplanted on to 7.6-L black plastic pots filled with a mixture of perlite and vermiculite with a volumetric ratio of 1:1. Mean, maximum, and minimum daytime air temperatures, determined during sample fruit growth from 21 Aug. to 9 Oct. 2008, were ≈24, 26, and 21 °C, respectively. Mean, maximum, and minimum nighttime temperatures during this period were ≈22, 24, and 20 °C, respectively. Daily mean relative humidity was maintained above 65%. Daily *PPF* integral on sunny days was between 15 and 20 $mol \cdot m^{-2} \cdot d^{-1}$.

For both experiments, the nutrient solution was supplied using a drip irrigation system with a feeding rate of 100 to 120 mL per irrigation event per plant. The irrigation frequency and irrigation period in a day were adjusted depending on plant growth so as to maintain a minimum of 30% efflux of the nutrient solution and to avoid salt accumulation. Based on plant growth, the irrigation frequency was increased from once per 60 min to once per 20 min and the irrigation period in a day was increased from 7.5 h to 9 h. The basal composition of the full-strength modified Hoagland nutrient solution except nitrogen (N) was prepared according to Wu and Kubota (2008) with slight modifications (Matsuda et al., 2009). The concentration of N, all derived from NO_3-N, was 190 and 100 $mg \cdot L^{-1}$ for '22.11.21' and '22.11.5', respectively. Common greenhouse plant maintenance, including pruning older leaves and removing side shoots, took place on a weekly basis. Flowering trusses were mechanically vibrated for 1 sec every other day using an electric device to promote pollination. Fruits were pruned to five and seven per truss for '22.11.21' and '22.11.5', respectively.

Fruit harvest. All fruits on each plant were harvested at one time on 13 Mar. 2008 and 9 Oct. 2009 for '22.11.21' and '22.11.5', respectively. Green fruits were selected based on the color classification for fresh tomatoes (United States Department of Agriculture, 1991), and the fresh weight (FW) and the longest diameter on the equatorial plane were measured. The fruits were then divided into three portions and FW of each portion was measured. The three portions were subjected to determination of dry weight (DW), TSP concentration, and F1-V concentration. Fruit DW was determined after it was oven-dried at 80 °C for at least 5 d and dry matter percentage was computed. Another portion for TSP determination was kept at –80 °C until analyses. The portion for F1-V determination was kept at –80 °C, freeze-dried for at least 72 h, pulverized to powder, vacuum-sealed, and stored until analyses.

Biochemical assay. Fruit TSP concentration was determined by Lowry-Folin assay (Lowry et al., 1951) as described in Matsuda et al. (2009). Calibration curves were made with bovine serum albumin. Fruit F1-V concentration was determined by enzyme-linked immunosorbent assay using a rabbit polyclonal anti-F1-V antibody as described in Alvarez et al. (2006).

Data analysis. Regression analysis was performed using statistical software (JMP; SAS Institute Inc., Cary, NC).

Results and Discussion

We defined fruit growth index (FGI) as an indicator of fruit growth during the green stage. In general, tomato fruit enlarge with time after anthesis during the green stage reach maximum size at around the end of the green stage and hardly change in size after the breaker stage through the red stage (Wu and Kubota, 2008). Here, we simply defined FGI for green fruits as their relative fruit diameter to the maximum diameter. The maximum diameter was estimated as the mean diameter of fruits between the breaker and red stages. Similar diameter-based evaluation of the physiological change in developing tomato fruit was also reported by Eltayeb and Roddick (1984). They showed almost the same pattern of the change in fruit alkaloid content irrespective of its expression based on fruit diameter or the day after anthesis. Note that because we use the average maximum diameter, FGI of an individual green fruit can exceed 1.0. The maximum diameters for '22.11.21' and '22.11.5' under the conditions of this study were 5.9 and 4.4 cm, respectively.

Whole-fruit FW increased exponentially as FGI increased during the green stage in both '22.11.21' and '22.11.5' (Fig. 1A). Whole-fruit DW also increased exponentially with increasing FGI (Fig. 1B). Fruit FW and DW were higher in '22.11.21' than in '22.11.5' at the same FGI. Dry matter percentage of the fruits decreased linearly during the green stage in both plants (Fig. 1C).

The TSP concentration per unit DW markedly decreased as FGI increased in both tomato lines (Fig. 2). When expressed on a whole-fruit basis, it appeared that there was no clear correlation between TSP content and FGI in '22.11.21' (Fig. 2). In '22.11.5', TSP content per whole fruit increased linearly with increasing FGI (Fig. 2).

Both F1-V concentration and F1-V content were higher in '22.11.5' than in '22.11.21' throughout the green stage (Fig. 3). In '22.11.21', F1-V concentration rapidly decreased at the beginning of the green stage and reached almost the minimum level at FGI of 0.8. The F1-V concentration decreased as FGI increased also in '22.11.5', but the pattern of the decrease was linear and different from that in '22.11.21'. The F1-V content per fruit decreased linearly with increasing FGI in '22.11.21'. In '22.11.5', the F1-V content per fruit tended to decrease after FGI of 0.6 if one exceptional datum is excluded, which was the highest F1-V content observed at the late green stage (FGI ≈1.1).

Based on these observations with the two transgenic plants, we propose the collection of small green fruits between 0.3 and 0.6 FGI (gray area in Fig. 3) without pruning fruits on the truss as a method for maximizing the F1-V yields. The F1-V content per whole fruit in young green fruits (FGI = 0.3 to 0.6) was similar to or even higher than that in mature green fruits (FGI > 0.6) (Fig. 3). Therefore, by harvesting young fruits without pruning, the number of fruits might be increased compared with the common harvesting practice without

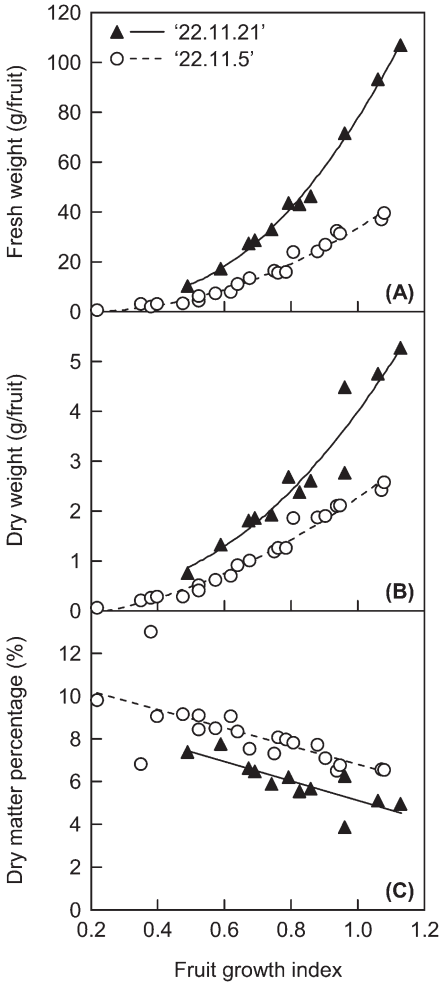

Fig. 1. Changes in tomato fruit fresh weight (**A**), dry weight (**B**), and dry matter percentage (**C**) of ‘22.11.21’ (filled triangles and solid line) and ‘22.11.5’ (open circles and broken line) as a function of fruit growth index. See Table 1 for regression equations and coefficients of determination.

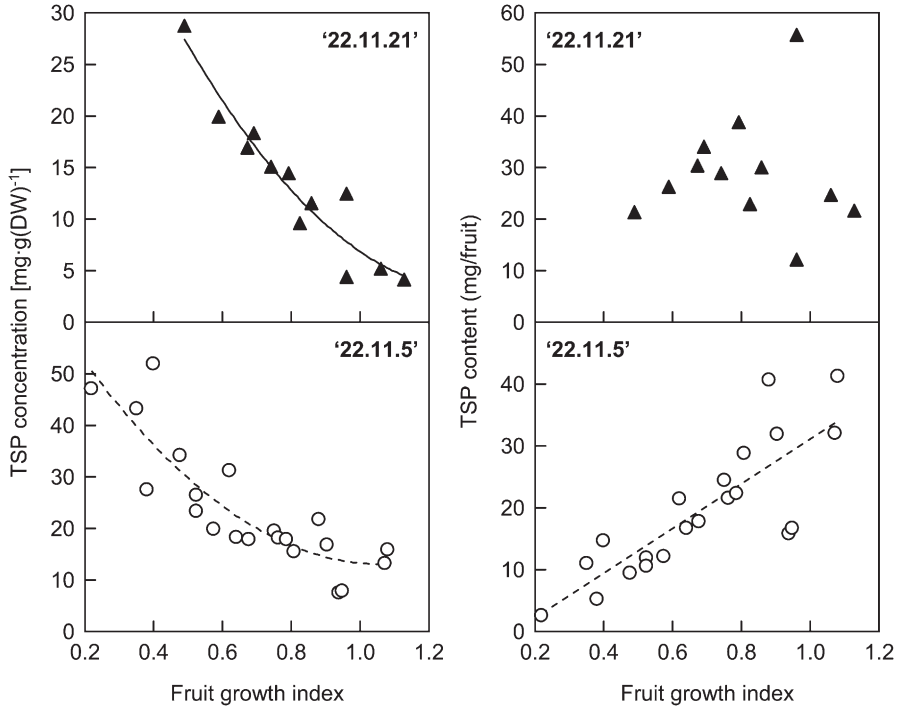

Fig. 2. Changes in tomato fruit total soluble protein (TSP) concentration per unit dry weight (DW) (left panels) and TSP content per fruit (right panels) of ‘22.11.21’ (top panels) and ‘22.11.5’ (bottom panels) as a function of fruit growth index. See Table 1 for regression equations and coefficients of determination.

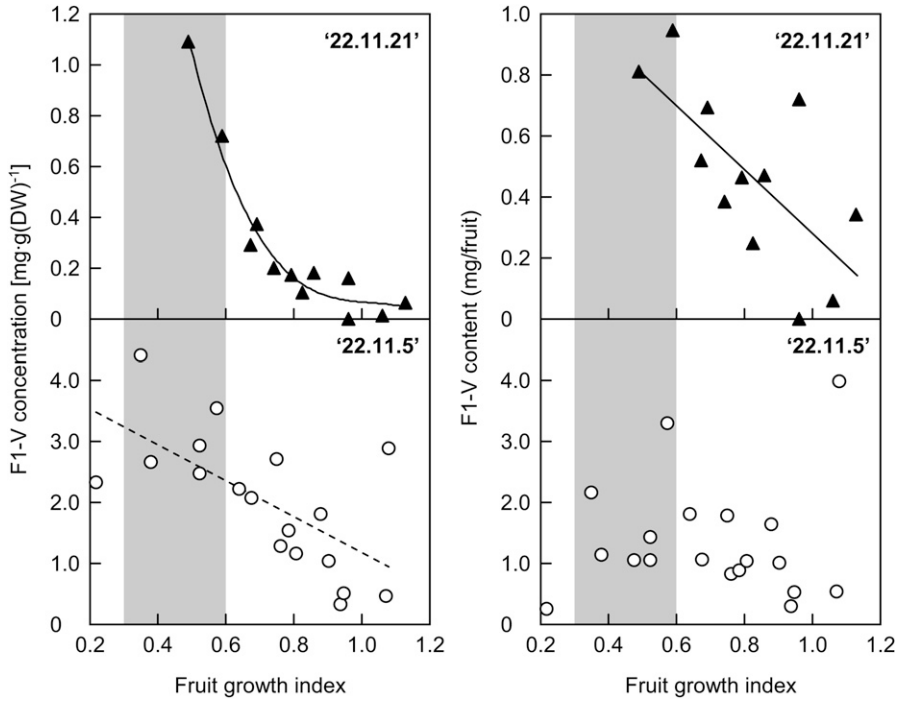

Fig. 3. Changes in tomato fruit F1-V concentration per unit dry weight (DW) (left panels) and F1-V content per fruit (right panels) of ‘22.11.21’ (top panels) and ‘22.11.5’ (bottom panels) as a function of fruit growth index. Gray area indicates an optimal timing of harvest for those transgenic lines (see text). See Table 1 for regression equations and coefficients of determination.

decreasing F1-V content per fruit, which we expect would result in higher total F1-V production per truss. In addition, young green fruits had higher F1-V concentrations than mature green fruits in both plants (Fig. 3). This higher F1-V concentration is advantageous, because it could contribute to producing an efficacious immunogenic oral vaccine in freeze-dried, powdered form with an appropriate, feasible mass.

Although we did not measure the actual harvest date after anthesis for an individual fruit of the transgenic plants, a fruit at 0.3 to 0.6 FGI was estimated to be approximately equivalent to a 7- to 14-d-old fruit after anthesis. This estimation was based on Figure 1A and the time course of fruit FW in wild-type ‘Durinta’ (Wu and Kubota, 2008), which requires a similar period from anthesis to maximal green fruit to our F1-V transgenics under the same growth conditions (data not shown).

The reason why the two transgenic plants showed different levels and trend of the changes in TSP and F1-V (Figs. 2 and 3) as well as in fruit FW and DW (Fig. 1) was not determined. In both plants, TSP and F1-V concentrations decreased with increasing FGI (Figs. 2 and 3), but the absolute levels and the rate of decrease differed. The parental T_1 plant of ‘22.11.21’ and ‘22.11.5’, ‘22.11’, carried two copies of *f1-v* (Alvarez et al., 2006), and the T_2 plant population, including ‘22.11.21’ and ‘22.11.5’, can be a segregating generation at each of two *f1-v* loci (Matsuda et al., 2009). Although we did not determine the *f1-v* gene copy number in ‘22.11.21’

and '22.11.5' directly, the plants were probably genetically different. Also, the plants were grown using different growing media (rockwool versus vermiculite/perlite) and in different seasons with different physical environments, especially light intensity and temperature that might affect the rates of decrease in F1-V and TSP concentrations. The larger fruit size in '22.11.21' than in '22.11.5' may have been a result of being pruned to five fruits per every truss, whereas '22.11.5' was pruned to seven fruits per every truss. In general, final fruit size decreases with increasing fruit number per truss in tomato (Heuvelink, 2005). Nevertheless, irrespective of these differences, collecting small green fruits without pruning fruits on the truss would probably be effective for both transgenic plants.

In both plants, positive correlations were found between F1-V and TSP concentrations (Fig. 4A–B), suggesting that the decrease in overall soluble protein concentration during fruit growth is one factor that brings about the decrease in F1-V concentration. However, in neither '22.11.21' nor '22.11.5' was a clear correlation observed between whole-fruit F1-V and TSP contents (Fig. 4C–D). The reason why there was a correlation only in the concentration-based relationship but not in the whole fruit-based relationship was not known, and the future investigation of the background biochemistry has to be done. In addition, in the concentration-based relationship for '22.11.21' (Fig. 4B), the coefficient of determination (r^2) was not as high (0.509) as '22.11.21' (0.941). Thus, the decrease in F1-V concentration was not solely accounted for by the decrease in TSP concentration, suggesting other factors are involved. It is largely uncertain how the balance between synthesis and degradation of a foreign protein changes during fruit growth and ripening of tomatoes. A transgene driven by the CaMV 35S promoter is expressed similarly in young green fruits, mature green fruits, and red fruits in a transgenic tomato plant (Sun et al., 2007). The change in transcriptional activity of *f1-v* in the F1-V transgenic tomato, which was also driven by the CaMV 35S, is to be addressed in future work. On the other hand, even when expressed on a whole-fruit basis, F1-V content in '22.11.21' decreased with fruit growth. The same trend was not clear in '22.11.5' grown under different conditions (Fig. 3). Recently, researchers have pointed out that degradation of foreign proteins by proteolysis in plant tissues could potentially lead to low foreign protein yield (Benchabane et al., 2008; Doran, 2006). F1-V degradation by endogenous proteases during fruit growth in the transgenic tomatoes should be investigated in future studies.

In summary, F1-V concentration of transgenic tomato decreased as fruit enlarged during the green stage. The F1-V content per whole fruit in young green fruits was similar to or even higher than that in mature green ones. We propose collecting small green fruits without pruning fruits on the truss as a harvest practice for maximizing both F1-V yield and concentration simultaneously. The uniqueness of this finding is that the optimum protocols of plant management and harvesting for PMP production can be substantially different from those currently used in fruit production in commercial hydroponic greenhouses. Our findings would provide important

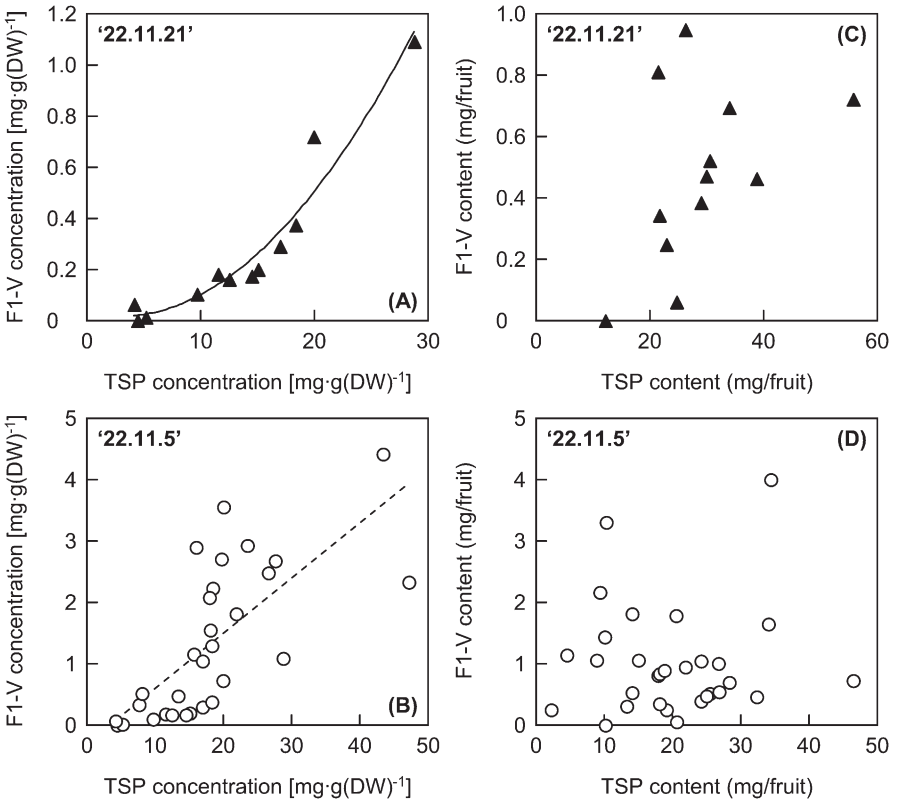

Fig. 4. The relationships between tomato fruit F1-V and total soluble protein (TSP) concentrations per unit dry weight (DW) (left panels) and between F1-V and TSP contents per fruit (right panels) in '22.11.21' (top panels) and '22.11.5' (bottom panels). See Table 1 for regression equations and coefficients of determination.

Table 1. Regression equations and coefficients of determination (r^2) for the relationships between fruit characteristics in '22.11.21' and '22.11.5'.

Figure	Dependent variable (y)	Independent variable (x)	Plant	Regression equation	r^{2z}
1A	Fresh weight	FGI[y]	22.11.21	$y = 49.8x^2 - 18.1x + 1.76$	0.980*
			22.11.5′	$y = 158x^2 - 103x + 23.4$	0.994*
1B	Dry weight	FGI	22.11.21	$y = 2.18x^2 + 0.298x - 0.210$	0.975*
			22.11.5′	$y = 5.80x^2 - 2.50x + 0.704$	0.919*
1C	Dry matter percentage	FGI	22.11.21	$y = -4.55x + 9.67$	0.669*
			22.11.5′	$y = -4.27x + 11.1$	0.488*
2	TSP[x] concentration	FGI	22.11.21	$y = 34.4x^2 - 91.6x + 64.1$	0.914*
			22.11.5′	$y = 52.9x^2 - 112x + 72.6$	0.770*
	TSP content	FGI	22.11.5′	$y = 36.1x - 5.00$	0.677*
3	F1-V[w] concentration	FGI	22.11.21	$y = -6.39x^3 + 19.7x^2 - 20.3x + 7.09$	0.965*
			22.11.5′	$y = -2.94x + 4.12$	0.428*
	F1-V content	FGI	22.11.21	$y = -1.05x + 1.33$	0.482*
4A	F1-V concentration	TSP concentration	22.11.21	$y = 0.00165x^2 - 0.00921x + 0.0290$	0.941*
4B	F1-V concentration	TSP concentration	22.11.5′	$y = 0.0897x - 0.290$	0.509*

[z]An asterisk (*) represents the significance of the regression by the test of no correlation at $P < 0.05$.
[y]Fruit growth index.
[x]Total soluble protein.
[w]An antigen fusion protein and a candidate subunit vaccine against plague.

information for commercial development of PMP production.

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