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Chlorophyll fluorescence imaging can reflect histological development of vascular connection in grafting union in some Solanaceae species

C. PENELLA[*], A. PINA[**], A. SAN BAUTISTA[***], S. LÓPEZ-GALARZA[***] and Á. CALATAYUD[*,+]

[*]*Instituto Valenciano de Investigaciones Agrarias (IVIA). Departamento de Horticultura, Valencia, Spain*

[**]*Centro de Investigación y Tecnología Agroalimentaria de Aragón, Unidad de Hortofruticultura, Zaragoza, Spain*

[***]*Universitat Politècnica de València. Departamento de Producción Vegetal, Valencia, Spain.*

[+]Corresponding autor; Dr. Ángeles Calatayud, phone: +34 3424039, fax: +34 3424000, e-mail: *calatayud_ang@gva.es*

Abstract

Graft union development in plants has been studied mainly by destructive methods like histological studies. The aim of this work was to evaluate whether the chlorophyll fluorescence imaging (CFI) technique is enough sensitive to reflect the changes at the cellular level from different Solanaceae grafted plants 30 days after grafting, when both graft partners were well fused and strong enough in all plant combinations. The pepper cultivar 'Adige' was grafted onto different *Capsicum* spp. accessions typified with different compatibility degrees; eggplant grafted on *Solanum torvum* and pepper homografts like high compatibility; pepper grafted on *S. torvum* and pepper grafted on tomato like incompatibles. 'Adige'/'Adige' and 'Adige'/pepper A25 showed a higher F_v/F_m value associated with higher values of Φ_{PSII} and q_P as well as with vascular regeneration across the graft interface. The results obtained highlight that CFI monitoring changes in chlorophyll fluorescence parameters reflect histological behaviour measurements in grafted Solanaceae plants.

Additional key words: callus cells; graft (in)-compatibility; pepper, photochemical quenching; vascular connections.

Abbreviations: CFI – chlorophyll fluorescence images; DAG – days after grafting; F_m – maximal fluorescence yield of the dark adapted samples; F_m` – maximal fluorescence yield of the light adapted samples; $F_o$ – minimal fluorescence yield of the dark adapted samples; $F_o$`– minimal fluorescence yield of the light adapted samples; F_s – steady-state fluorescence yield during actinic irradiation; F_v – variable fluorescence (F_m – F_o) in the dark adapted samples; F_v/F_m – maximum quantum efficiency of PSII photochemistry; NPQ – nonphotochemical quenching calculated

from Stern-Volmer equation; q_P – photochemical quenching; Φ_{PSII} – actual quantum efficiency of PSII.

Introduction

Grafting can be defined as the natural or deliberate fusion of plant parts so that vascular continuity is established between them and the resulting genetically composite organism functions as a single plant (Mudge *et al.* 2009). Grafting is a technique that has been widely used for centuries in woody plants. Nowadays, this technique is being greatly expanding in vegetables plants particularly in *Solanaceae* and *Cucurbitaceae* families, to reduce pathogens infections (Biles *et al*. 1989; Padgett and Morrison 1990) or to increase resistance to abiotic stresses, such as drought (Sánchez-Rodríguez *et al*. 2013, Penella *et al*. 2014a) , salinity (Orsini *et al*. 2013, Penella *et al*. 2015), or heavy metals (Savvas *et al*. 2010). This is also used to enhance nutrient uptake (Ruiz *et al*. 1997) or to increase yields and fruit quality (Rouphael *et al*. 2010, Penella *et al*. 2013).

During the graft union formation between rootstock and scion, many researchers have observed callus proliferation (from both the rootstock and the scion), callus bridge formation, differentiation of cambium tissue from callus cells and the production of secondary xylem and phloem (Hartmann *et al*. 2002, Pina and Errea 2005, Aloni *et al*. 2010, Trinchera *et al*. 2013). A low or incorrect callus formation between the rootstock and scion could lead to defoliation, reduction of scion growth and low survival of grafted plants (Kawaguchi *et al*. 2008, Johkan *et al*. 2009) reducing water flow to shoots (decreased hydraulic conductance) (Martínez-Ballesta *et al*. 2010).

There is no precise definition of graft compatibility and generally means the establishment of a successful graft union as well as extended survival and proper

functioning of the composite rootstock-scion (Goldschmidt 2014). Graft incompatibility may be defined as failure to form a successful graft union. A lack of, or decrease in number of differentiated vascular bundles, or the dysfunction of differentiated vascular bundles at the graft union has been reported to inhibit transport of nutrients to scion (Wang and Kollmann 1996, Schöning and Kollmann 1997). Characterization of incompatibility is not a simple process because graft combinations can initially unite with apparent success, but gradually develop incompatibility symptoms with time, due to either a limited and/or not fully functional vascular reconnection between scion and rootstock at the graft interface which causes the subsequent failure of the graft union (Errea *et al*. 1994, Errea *et al*. 2001) or the development of abnormal growth patterns (Kawaguchi *et al*. 2008).

The major causes implicated in graft incompatibility in *Solanaceous* crops are anatomical and/or biochemical (Deloire and Hébant 1982, Ives *et al*. 2012). In severely incompatible grafted plants such as pepper/tomato or pepper/eggplant grafts growth inhibition and high mortality was observed due to narrow and irregular xylem connections between scions and rootstocks; this was associated with higher concentration of sugars and starch above than below the graft union (Kawaguchi et al., 2008). Elevated production of reactive oxygen species, decrease in antioxidant enzymes activities or increase in polyphenols metabolites are a well-documented fact in graft-incompatible combinations from different *Solanaceous* species (Ives *et al*. 2012, Deloire *et al.* 1982, Fernández-García *et al.* 2004a)

Pepper (*Capsicum annuum*) is grown in most countries of the world, with 1.93 million of ha cultivated area and is one of the most important crops in Mediterranean area. Grafted pepper plants are used to cope with biotic and abiotic stresses (Penella *et al.* 2014a, Penella *et al.* 2015, Oka *et al.* 2004). Peppers have been described as

compatible only with other *Capsicum* species but not with all of them. In this sense, Otsuka (Otsuka 1957) reported that tomato/pepper or pepper/tomato graft combinations were completely incompatible because plant growth was severely suppressed, in contrast with other *Solanaceae* species like tomato or eggplant, which are able to be grafted onto some different species within their family (Deloire and Hébant 1982, Miguel *et al*. 2007, Kawaguchi *et al*. 2008, Ives *et al*. 2012).

The first methods used to predict graft incompatibility relied on external symptoms such as swollen union, death or decline in vegetative growth and vigour of the scion, and marked differences in growth of both scion and rootstock (Otsuka 1957). Afterwards, physiological and anatomical methods for the diagnosis of graft (in)-compatibility have been developed, such as the measurement of peroxidase and catalase concentrations as the enzymes implicated in graft development (Fernandez-Garcia *et al*. 2004a); the hormone levels (Yin *et al*. 2012); reactive oxygen species (ROS) production (Irisarri *et al*. 2015); accumulation of sugars (Kawaguchi *et al*. 2008), hydraulic root conductivity (Clearwater *et al*. 2004) or histological measurements (Pina *et al*. 2012). However, all these methods are invasive (destructive), slow and/or most of them are thought to woody plants.

The use of X-ray tomography to visualize the 3D structure of the graft union (Milien *et al*. 2012) is a non-destructive method to evaluate internal structure in the graft area, but the potential impact of the ionizing effects of the X-rays on the living tissue can be negative, as it has been demonstrated in a growth inhibited *Arabidopsis* seedling (Dhondt *et al*. 2010) and consequently has to be considered.

Another nondestructive method without effects on the plant tissues and on the subsequent development of the plant is the use of the chlorophyll fluorescence imaging (CFI). CFI has been used to predict compatibility in melon grafted plants

(Calatayud *et al*. 2013), and the use of images for monitoring florescence parameters allowed visualize possible alterations in grafted plants (Quilliam *et al*. 2006; Calatayud *et al*. 2013). This could be an intuitive, quick and noninvasive method providing detailed information on spatial and temporal heterogeneity for evaluating behaviour in grafted plants.

The aim of this work was to evaluate the potential of CFI in different Solanaceae plant combinations using positive controls (pepper grafted onto pepper) and negative controls (tomato/pepper and eggplant/pepper), connecting values of CFI parameters to histological observations in order to demonstrate whether or not CFI can reflect the morphological and anatomical at the graft interface between both graft partners. To reach this objective, the commercial pepper cultivar 'Adige' was grafted on different *Capsicum* spp. accessions typified with different compatibility degrees in terms of yield and quality in previous works performed by this research group (Penella *et al*. 2013; Penella *et al*. 2014b; Penella *et al*. 2014c; Penella *et al*. 2015) and also used different graft combinations with known graft compatibility as controls: eggplant grafted on *S. torvum* and pepper homografts (high compatibility), pepper grafted on *S. torvum* and pepper grafted on tomato like incompatible unions.

Materials and methods

Plant materials and grafting plants: A total of nine combinations of plants were evaluated for graft compatibility. Cultivar 'Adige' *Capsicum annuum* L. (Lamuyo type; Sakata seeds, Japan – code A), was grafted onto the accessions of *C. annuum* L. – code A25 and code A5, *Capsicum chinense* Jacq. – code C12, *Capsicum baccatum* L. var. *pendulum* – code B14 used in previous studies on physiological and

agronomical responses that showed different compatibility degree (Penella *et al*. 2013, Penella *et al*. 2014a, Penella *et al*. 2014c, Penella *et al*. 2015). In addition, two commercial rootstocks were used: *Solanum torvum* Sw. "Torvum vigor" (Ramiro Arnedo, Spain – code ST) and *L. esculentum* x *L. hirsutum* "Beaufort" (De Ruiter Seeds, Nederland – code BEU) described in the bibliography as incompatible (Kawaguchi *et al*. 2008). Besides tomato var. Gordal (Mascarell seeds, Spain – code TOM) was grafted on ST – TOM/ST, this combination has been described as moderately incompatible (Kawaguchi *et al*. 2008). *Solanum melongena* L. eggplant "Cristal" (Fitó seeds, Spain – code EGG) was also grafted onto ST– EGG/ST and selfgrafted plants of 'Adige'– A/A were used as positive controls.

As previously mentioned, plant combinations and their codes used in histological and chlorophyll fluorescence measurements. Between bracket are identify the intra or interspecific grafting for each plant combinations. Estimated affinity (according to literature and previous studies) is represented by (++) – compatible, (+) – moderately compatible, (-) – moderately incompatible and (--) – incompatible grafted plant combinations.

Rootstock (code)	Scion	Graft plant	Estimated affinity
Capsicum annuum L. var. Adige (A)	Adige (A)	A/A (intraspecific)	++
C. annuum (A25)	Pepper var. Adige (A)	A/A25 (intraspecific)	++
C. annuum (A5)	Pepper var. Adige (A)	A/A5 (intraspecific)	-
C. *baccatum* (B14)	Pepper var. Adige (A)	A/B14 (intraspecific)	+
C. *chinense* (C12)	Pepper var. Adige (A)	A/C12 (interspecific)	+
S. torvum (ST)	Eggplant var. Cristal (EGG)	EGG/ST (interspecific)	++
S. torvum (ST)	Pepper var. Adige (A)	A/ST (interspecific)	--
S. torvum (ST)	Tomato var. Gordal (TOM)	TOM/ST (interspecific)	-
Tomato Beaufort (BEU)	Adige (A)	A/BEU (interspecific)	--

Plants were sown on 15th January 2014 in 104-cell polystyrene trays filled with peat-based substrate and kept under a Venlo-type glasshouse. The plants were transplanted to 54-cell trays. The different graft combinations were performed on 21th

March using the tube grafting method (cutting the growing tip of the rootstock at a 45º angle above the cotyledons, and fixing the rootstock and scion with a clip) (Penella *et al*. 2013).

Ten days after grafting, only the compatible grafted plants were fused about 75%, the number of fused in these plants increase with time, reaching 98% 20 days after grafting – DAG. In incompatible grafted plants, both graft partners were fused later on (30 DAG) and enough numbers of these plants were obtained for doing the measurements (30%) at this time. For this reason, a kinetic cannot done at earlier stages of development and all plants were measured at 30 days after grafting.

Light microscopy: The graft interfaces were fixed 30 days after grafting (DAG) in 3% glutaraldehyde in 50 mM Sorensen buffer (28.5% KH_2PO_4 50 mM and 71.5% Na_2HPO_4 mM.) at pH 7.2 for 2-h. After that, plant material was washed four times during 15 min in the same buffer. After infiltration in LR white resin:ethanol (1:2 v/v, 1:1 v/v, 2:1 v/v) for 60 min per stage, the specimens were embedded in historesin LR white overnight (London Resin Co., Woking, Surrey, UK) at 4ºC according to Tadeo *et al*. (Tadeo *et al*. 1997), and transversally sectioned at 2 µm using glass knives in a Leica RM 2165 Rotary Microtome (Leica Instruments, Heidelberg, Germany). The sections were stained in 0.05% toluidine blue 0 (Cl 52040, Merck, Darmstad, Germany) (O'Brien and McCully 1981), desiccated and mounted in Eukitt Mounting Medium 15322 (Electron Microscopy Sciences, Hatfield, PA, USA). Representative sections of three tissue samples per plant from ten plants were viewed under a Leitz Ortholux II fluorescence microscope (Leitz, Wetzal, Germany) operating in an optical mode and the images were captured with a Leica DC300 camera.

Chlorophyll fluorescence imaging: Chlorophyll fluorescence imaging measurements of grafted plants were performed 30 DAG from 15-20 plants per combination at 2 cm above and below the graft interface and the graft interface using an imaging-PAM fluorometer (Walz, Effeltrich, Germany). All plants were placed in the dark for 20 min prior to measurement. Images and values of minimum Chl fluorescence yield in the dark-adapted state, F_o, were determined using light pulses at low frequency (1 Hz). Maximum fluorescence Fm was determined by applying a blue saturation pulse (10 Hz). The maximum quantum yield of PSII photochemistry (F_v/F_m ratio) was determined as $(F_m - F_o)/F_m$ and images were captured. Actinic illumination (260 μmol m^{-2} s^{-1}) was then switched on and saturating pulses were applied at 20 s intervals for 5 min to determine $F_m`$ and Chl fluorescence kinetics during actinic illumination ($F_s$). The actual quantum efficiency of PSII photochemistry [$\phi_{PSII} = (F_m` - F_s)/F_m`$] (Genty *et al*. 1989), photochemical quenching [$q_P = (F_m` - F_s)/(F_m` - F_o`$] (Schreiber *et al*. 1986) and the non-photochemical quenching [$NPQ = F_m` - F_s/F_m`$] (Bilger and Björkman 1991) were calculated. The value of $F_o`$ was estimated using the approximation of (Oxborough and Baker 1997), [$F_o` = F_o/(F_v/F_m + F_o/F_m`$]. Three areas of measure were definite through PAM-software in stem of the plants (graft area, the rootstock and the scion). Fluorescence parameter values of all pixels within each area were averaged. Each value in the tables is the mean of the corresponding area of all samples (obtained from 15–20 different plants). Figure 2 shows the images of only a single plant (representative plant). Further information on CFI measurements can be obtained from (Calatayud *et al*. 2008; Calatayud *et al*. 2013).

Statistical analysis: One-way ANOVA was performed (Statgraphics Centurion XVI for Windows, Statistical Graphics Corp.) to compare the means of the fluorescence parameters. Mean separations were performed when significant differences were found using the least significance difference at *P<0.05*.

Results and discussion

Histological evaluation of scion/rootstock interactions: Table insert in materials and methods summarizes the plant codes used for histological and CFI studies. Pepper homografting (A/A) and the use of the intra-specific grafts (rootstock and scion belonging to the same botanical species) rootstocks B14, C12 and A25 showed a higher yield (Penella *et al*. 2014a; Penella *et al*. 2015) than the intra-specific combination 'Adige' grafted onto the rootstock A5 (A/A5). A/A5 combination had a lower growth than other grafted plants (A/B14, A/C12 and A/A25) and its stem diameter at the graft union was approximately three-fold greatest and provided lower fruit yields (Penella *et al*., 2013, Penella *et al*. 2014a).

The cellular events that led to a successful graft union include adhesion of the two graft partners, callus cell proliferation at the graft interface and cross-bridge formation of the vascular bundles to establish a functional vascular connection (Aloni *et al*. 2010, Goldschmidt 2014, Mudge *et al.* 2009, Pina and Errea 2005). Nevertheless, incomplete or nonfunctional vascular connections impede the vital upward and downward whole plant transfer routes, which might result in a dieback of the graft. By 30 DAG, a well developed vascular graft union was observed in the pepper homografts (A/A) and intraspecific heterografts eggplant grafted on *Solanum torvum* (EGG/ST) (Fig. 1*A*, B) and 'Adige' grafted in the pepper rootstock accessions A25

and C12 (Fig. 1*C, D*). In these combinations, most of the necrotic layer was absorbed at this stage and group of small callus cells are clustering resembling symplastic domains which is a prerequisite to begin more vascular differentiation (Pina *et al*. 2009). Higher levels of vascular differentiation were observed in the combination A/A25 (Fig. 1*C*) than in the combination A/C12 (Fig. 1*D*). In all combinations, cluster of callus cells were associated with the cut ends of the xylem from which them were derived and filled the graft interface. 'Adige' grafted on rootstock accession A/B14 showed a high cellular activity at the graft interface and callus cells bridging the two graft partners (Fig. 1*E*). Some developing tracheid elements were observed but not completely new xylem and phloem formation was displayed across the graft union 30 DAG. Similar anatomical results were obtained when grafted tomato onto *Solanum torvum* (TOM/ST) (more distantly taxonomic species) (Fig. 1*F*), indicating that the compatibility behaviour of both graft combinations (A/B14 and TOM/ST) was similar, moderately compatible, as reported by Kawaguchi *et al*. (2008) for TOM/ST.

A stronger level of graft incompatibility was observed when pepper cv. 'Adige' was grafted onto rootstock accession A5 (A/A5). In this case, histological examination provided clear evidence of discontinuous xylem elements in the graft union as well as large areas of unbroken necrotic lines along the wounded edges of the rootstock and the scion (Fig. 1*G*). This result was consistent with the anatomy of the severely incompatible union tomato/pepper (A/BEU) (Fig 1*H*). In addition, 'Adige' grafted on *S. torvum* (A/ST) produced weak unions, characterized by limited fusion between both graft partners (Fig. 1*I*) and the presence of cells enriched with green material inside the vacuoles similar to phenolic compounds, that are involved in the incompatibility reaction inhibiting division, development and differentiation into new tissues during the graft union formation (Errea 1998, Pina *et al*. 2012, Hudina *et al*. 2014).

In these three combinations A/A5, A/BEU and A/ST, the rootstock and scion tissue produced new vascular elements as well, but these did not cross the scion/rootstock border and therefore no graft union was formed. In incompatible heterografts between Arabidopsis grafted on tomato rootstock, it was reported that the remaining necrotic layer that developed at the graft interface seemed to inhibit the differentiation of vascular tissue across the graft union, either directly or indirectly, and thus prevented full vascular graft union formation between the two plants, since neither vascular bridge nor full graft union was visible (Flaishman *et al*. 2008). Other studies also reported the presence of narrow and irregular xylem elements in incompatible tomato/pepper heterografts (Kawaguchi *et al*. 2008, Ives *et al*. 2012).

Chlorophyll fluorescence imaging in grafted plants: The same grafted plants combinations used for histological evaluation were analysed by CFI.

In table 1, the mean values of F_v/F_m ratio for rootstock, scion and graft area of the nine plant combinations are shown. The F_v/F_m is one of the most common fluorescence parameter, as it is an indicator of plant stress (Rolfe and Scholes 2010) and reflects the maximal efficiency of excitation capture of dark-adapted plants and is correlated with the number of functional PSII reaction centres (Oquist and Chow 1992). Attending to F_v/F_m values in the rootstock area, four groups of plants can be distinguished according to ANOVA analyse: A/A, A/A25, A/B14, A/C12, TOM/ST and EGG/ST showed the higher F_v/F_m values, A/A5 with intermediate value, followed of the combination A/BEU and with lower F_v/F_m value A/ST. In compatible tomato grafted plants observations of the structure of graft union showed formation of xylem and phloem vessels through the graft union 8 DAG (Fernández-García *et al*. 2004b). But narrow and irregular connections were observed in graft union between

294 incompatible graft plants as tomato/pepper or pepper/tomato 3 weeks after grafting
295 (Kawaguchi *et al*. 2008). CFI measurements were performed at 30 DAG, therefore
296 the anatomical symptoms associated with graft (in)-compatibility has been already
297 internally manifested. The lower F_v/F_m ratio in rootstocks areas have been measured
298 in incompatible heterografted plants A/BEU and A/ST. As reported by the histological
299 study, a weak graft connection occurs in these plants combinations, in such a way
300 that it is expected that the translocation of assimilate from scion to the rootstock
301 result in higher carbohydrate concentration in the scion part and lower concentration
302 in the rootstocks (Kawaguchi *et al*. 2008). A limited assimilate supply to the
303 rootstocks could reduce the size of root system and decreased metabolic activity
304 increasing damage to the photosynthetic apparatus and decreasing F_v/F_m in
305 rootstock area. Likewise, the F_v/F_m values in the graft area followed the same
306 tendency showed by the rootstock area, but the values underwent an important
307 decrease for the incompatible grafts A/ST and A/BEU. It is probably a consequence
308 of the weak connection between rootstocks (*S. torvum* and tomato). A low or
309 incorrect callus formation lead to a bad vascular connection at the rootstock-scion
310 graft interface affecting water and nutrient translocation that can alter the
311 photosynthesis behaviour in the graft zone (Martínez-Ballesta *et al*. 2010). For this
312 reason, F_v/F_m values decreased to a greater extent compared with rootstocks values.
313 These insufficient connections of vascular bundles were reflected in the scion part
314 with lowest F_v/F_m values in ST/A and BEU/A (Table 1). F_v/F_m images of
315 representative's samples (Fig. 2) allowed visualize the rootstock, graft and the scion
316 areas, indicating that the technique is able to display large areas of graft zones. The
317 observation of color changes (ranging from black (0.000) to pink (1.000) revealed
318 spatial changes in the F_v/F_m images. In A/A, A/A25, A/B14, A/C12, TOM/ST and

EGG/ST different intensities of blue colors were observed associated with higher values of F_v/F_m. In A/A5 a black line was observed across graft area-scion indicating a null F_v/F_m values. A dramatic change in colors from blue-green and brown of F_v/F_m in A/ST and A/BEU were observed, that correspond with lower F_v/F_m values. It should be noted that the scion area in A/ST and A/BEU showed the colors green and brown associated with lowest F_v/F_m values.

When F_v/F_m values were compared at the scion from the different graft unions, the decrease in incompatible unions were more marked. Four categories could also be well definite: compatible plants (A/A, A/A25, A/B14, A/C12, TOM/ST and EGG/ST), moderate compatible A/A5 and incompatible A/TOM and strong incompatible A/ST. If a weak graft connection occurs in A/A5, A/TOM and A/ST, the probability of nutrient uptake reaching the scion decrease, leading to alteration of PSII photochemistry (Calatayud *et al*. 2013). In order to study the cause of this noticeable decline in F_v/F_m at the scion area, we analysed their photochemical and non-photochemical processes (Table 2). Statistical analysis of photochemical allowed differentiating four groups: A/A, A/A25 and EGG/ST with higher values of Φ_{PSII} and q_P; A/B14, A/C12 and TOM/ST with moderate decrease of photochemical processes; A/A5 with considerable decrease and the last group with the plant combinations A/ST and A/BEU with the lowest photochemical values. The decrease in Fv/Fm for the graft combinations A/A5, A/BEU and A/ST (Table 1) could be the result of an increase in protective nonradiative energy dissipation, photodamage of PSII centres or both (Osmond 1994). Inasmuch as NPQ is believed to indicate the capacity for photoprotective process (Osmond 1994), the decline in F_v/F_m ratio was attributable to PSII stress, because NPQ was adversely affected in scion areas for the three plant combinations (Table 2). In severely damaged tissues resulted in a decreased in NPQ

values (Berger *et al*. 2007). In addition, the lower q_P values (Table 2) observed in A/A5, A/BEU and A/ST indicated that their capacity for reoxidizing Q_A decreased, increased excitation pressure on PSII and contributed to the closure of PSII reaction centres. According with this result, the Φ_{PSII}, correlated with the quantum yield of non-cyclic electron transport (Genty *et al*. 1989), and was markedly reduced mainly in A/ST and A/BEU (Table 3). This reflect that a low or incorrect callus formation (Fig. 1) affected vascular connection in the rootstock/scion interface and may determine a decrease in water and nutrient translocation (Martínez-Ballesta *et al*. 2010) affecting photosynthesis performance limiting the availability of assimilate for plant growth.

In compatible and moderate compatibility grafted plants A/A, A/A25, A/B14, A/C12, TOM/ST and EGG/ST a higher Φ_{PSII} and q_P in scion area was observed. This increase in photochemical process in the scion can feed the new connections formation at the graft interface. Associated with an electron flow stimulated (Φ_{PSII}), NPQ increased as a protection mechanism in these plant combinations (Berger *et al*. 2007, Guidi *et al.* 2007).

Connecting values of CFI parameters to histological studies: Chlorophyll fluorescence imaging displayed the histological observations in our nine plant combinations. The statistical groups in Φ_{PSII} parameter measurement in the scion area reflected better the histological observations indicating that A/A, A/A25 and EGG/ST with highest values of photochemical parameter showed well vascular graft union with necrotic layer absorbed, and group of small callus cells are clustered resembling symplastic domains which is a prerequisite to begin more vascular differentiation. The group of A/B14, A/C12 and TOM/ST with moderate decrease of photochemical processes express well developed vascular graft union, but with less

vascular differentiation than the first group. Likewise, a strong correlation was observed between a considerable decrease in $\Phi_{PSII,}$ and the presence of discontinuous xylem elements in the graft union for the combination A/A5, as well as large areas of unbroken necrotic lines along the wounded edges of the rootstock and the scion. The remaining combinations, A/ST and A/BEU, with the lowest photochemical values produced weak unions, characterized by limited fusion between both graft partners and the presence of cells enriched with green material inside the vacuoles similar to phenolic compounds, which are involved in the incompatibility reaction inhibiting division, development and differentiation into new tissues during the graft union formation (Errea 1998).

Conclusion: This study represents a satisfactory and wide calibration of the changes in CFI that may be useful as reflection and/or accompaniment of histological behaviour in grafted Solanaceae plants studies. In general terms, CFI provided information on graft stage and represents a quick, non-invasive technique that not requires sample preparation for studying union in vegetables. The main interest of CFI methods is associated with the images that permit large areas of graft zones to be viewed on the same plant overtime. However, CFI does not replace systematically classical histology in terms of understanding morphological and anatomical developments at the graft interface. CFI could represent a high-throughput phenotyping tool necessary to reduce the time invested for determining behaviour in grafted plants and could be used as a sensor in decision support systems for detection of graft compatibility

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References

Aloni B., Cohen R., Karni L., Aktas H., Edelstein M.: Hormonal signaling in rootstock-scion interactions. – Sci. Hortic. **127**: 119-126, 2010.

Berger S., Benediktyová Z., Matous K., Bonfig K., Mueller M.J., Nedbal L., Roitsch T.: Visualization of dynamics of plant-pathogen interaction by novel combination of chlorophyll fluorescence imaging and statistical analysis: differential effects of virulent and avirulent strains of *P. syringae* and of oxylipins on *A. thaliana*. – J. Exp.Bot. **58**: 797-806, 2007.

Biles C., Martyn R., Wilson H.: Isoenzymes and general proteins from various watermelon cultivars and tissue types. – HortSci. **24**: 810-812, 1989.

Bilger W., Björkman O.: Temperature dependence of violaxanthin de-epoxidation and non-photochemical fluorescence quenching in intact leaves of *Gossypium hirsutum* L. and *Malva parviflora* L. – Planta, **184**: 226-234, 1991.

Calatayud Á., Gorbe E., Roca D., Martínez P.F.: Effect of two nutrient solution temperatures on nitrate uptake, nitrate reductase activity, NH_4^+ concentration and chlorophyll a fluorescence in rose plants. – Environ. Exp. Bot. **64:** 65-74, 2008.

Calatayud Á., San Bautista A., Pascual B., Maroto J.V., López-Galarza S.: Use of chlorophyll fluorescence imaging as diagnostic technique to predict compatibility in melon graft. – Sci. Hortic., **149**: 13-18, 2013.

Clearwater M.J., Lowe R.G., Hofstee B.J., Barclay C., Mandemaker A.J.,

Blattmann P.: Hydraulic conductance and rootstock effects in grafted vines of kiwifruit. – J. Exp. Bot. **55**: 1371-82, 2004.

Deloire A., Hébant C.: Peroxidase activity and lignification at the interface between stock and scion of compatible and incompatible grafts of Capsicum on Lycopersicum. Ann. Bot. **49:** 887-891, 1982.

Dhondt S., Vanhaeren H., Van Loo D., Cnudde V., Inzé D.: Plant structure visualization by high-resolution X-ray computed tomography. – Trends Plant Sci. **15**: 419-22, 2010.

Errea P., Garay L., Marín J.A.: Early detection of graft incompatibility in apricot (*Prunus armeniaca*) using in vitro techniques. – Physiol. Plant. **112**: 135-141, 2001.

Errea P.: Implications of phenolic compounds in graft incompatibility in fruit tree species. – Sci. Hortic. **74**: 195-205, 1998.

Errea P., Felipe A., Herrero M.: Graft establishment between compatible and incompatible Prunus spp. – J. Exp. Bot. **45**: 393-401, 1994.

Fernández-Garcia N., Carvajal M., Olmos E.: Graft union formation in tomato plants: peroxidase and catalase involvement. – Ann. Bot. **93**: 53-60, 2004a.

Fernández-García N., Martínez V., Carvajal M.: Effect of salinity on growth, mineral composition, and water relations of grafted tomato plants. – J. Plant Nutr. Soil Sci. **167**: 616-622, 2004b.

Flaishman M. a., Loginovsky K., Golobowich S., Lev-Yadun S.: *Arabidopsis thaliana* as a model system for graft union development in homografts and heterografts. – J.Plant Growth Regul. **27**: 231-239, 2008.

Genty B., Briantais J.-M., Baker N.R.: The relationship between the quantum yield

of photosynthetic electron transport and quenching of chlorophyll fluorescence. – Biochem. Biophys. Acta (BBA) – General Subjects **990**: 87-92, 1989.

Goldschmidt E.E.: Plant grafting: new mechanisms, evolutionary implications.– Front. Plant Sci. **5**: 727, 2014.

Guidi L., Mori S., Degl'Innocenti E., Pecchia S.: Effects of ozone exposure or fungal pathogen on white lupin leaves as determined by imaging of chlorophyll a fluorescence. – Plant Physiol. Biochem. **45**: 851-857, 2007.

Hartmann H., Kester D., Davies F., Geneve R.: Plant propagation. Principes and practices seventh. Upper Saddle River, NJ: Prentice Hall. 2002.

Hudina M., Orazem P., Jakopic J., Stampar F.: The phenolic content and its involvement in the graft incompatibility process of various pear rootstocks (*Pyrus communis* L.). – J. Plant Physiol. **171**: 76-84, 2014.

Irisarri P., Binczycki P., Errea P., Martens H.J., Pina A.: Oxidative stress associated with rootstock-scion interactions in pear/quince combinations during early stages of graft development. – J. Plant Physiol. **176**: 25-35, 2015.

Ives L., Brathwaite R., Barclay G., Isaac W.A., Connor C.B.: Graft Compatibility of Scotch Bonnet. – J. Agric. Sci. Tech. **2**: 81-92, 2012.

Johkan M., Mitukuri K., Yamasaki S., Mori G., Oda M.: Causes of defoliation and low survival rate of grafted sweet pepper plants. – Sci. Hortic. **119**, 103-107, 2009.

Kawaguchi M., Taji A., Backhouse D., Oda M., Science R.: Anatomy and physiology of graft incompatibility in solanaceous plants. – J. Hortic. Sci. Biotech. **83**: 581-588, 2008.

Martínez-Ballesta M.C., Alcaraz-López C., Muries B., Mota-Cadenas C., Carvajal

M.: Physiological aspects of rootstock-scion interactions.– Sci. Hortic. **127**: 112-118, 2010.

Miguel A., De la Torre F., Baixauli C., Maroto J. V, Jordá M.C., López M.M., García-Jímenez J.: Injerto de hortalizas. Valencia, Spain: Ministerio de Agricultura, Pesca y Alimentación y Fundación Rural Caja. 2007.

Milien M., Renault-Spilmont A.-S., Cookson S.J., Sarrazin A., Verdeil J.-L.: Visualization of the 3D structure of the graft union of grapevine using X-ray tomography. – Sci. Hortic. **144**: 130-140, 2012.

Mudge K., Janick J., Scofield S., Goldschmidt E. E.: A history of grafting. – Hortic. Rev. (Am. Soc. Hortic. Sci.) **35**: 437-494, 2009.

O'Brien T., McCully M.: The study of plant structure: principles and selected methods. Melburne: Termacarphy. 1981.

Oka Y., Offenbach R., Pivonia S.: Pepper rootstock graft compatibility and response to *Meloidogyne javanica* and *M. incognita*. – J. Nematology **36**: 137-141, 2004.

Oquist G., Chow W.S.: On the relationship between the quantum yield of Photosystem II electron transport, as determined by chlorophyll fluorescence and the quantum yield of CO_2-dependent O_2 evolution. – Photosynth. Res. **33**: 51-62, 1992.

Orsini F., Sanoubar R., Oztekin G.B., Kappel N., Tepecik M., Quacquarelli C., Tuzel Y., Bona S., Gianquinto G.: Improved stomatal regulation and ion partitioning boosts salt tolerance in grafted melon. – Func. Plant Biol. **40**: 628-636, 2013.

Osmond C.B.: What is photoinhibition? Some insights from comparisons of shade

and sun plants. – In: Baker NR, Bowyer JR (ed.): Photoinhibition of Photosynthesis from Molecular Mechanisms to the Field. Bios Scientific Publishers.1994.

Otsuka K.: Studies on nutritional physiology of grafted plants 2. Rootstocks-scion influences on growth and on ionic accumulation of solanaceous grafts. – Jap. J.Soil Sci. Plant Nutr. **28:** 285-289, 1957.

Oxborough K., Baker N.R.: An instrument capable of imaging chlorophyll a fluorescence from intact leaves at very low irradiance and at cellular and subcellular levels of organization.– Plant Cell Environ. **20**: 1473-1483, 1997.

Padgett M., Morrison J.C. Changes in grape berry exudates during fruit development and their effect on mycelial growth of Botrytis cinerea. – J. Am. Soc. Hortic. Sci. **115**: 269-273, 1990.

Penella C., Nebauer S.G., Quiñones A., San Bautista A., López-Galarza S., Calatayud A.: Some rootstocks improve pepper tolerance to mild salinity through ionic regulation. – Plant Sci. **230**: 12-22, 2015.

Penella C., Nebauer S.G., Bautista A.S., López-Galarza S., Calatayud Á.: Rootstock alleviates PEG-induced water stress in grafted pepper seedlings: Physiological responses. – J. Plant Physiol. **171**: 842-851, 2014a.

Penella C., Nebauer S.G., López-Galarza S., Bautista A.S., Rodriguez-Burruezo A., Calatayud A.: Evaluation of some pepper genotypes as rootstocks in water stress conditions. – Hortic. Sci. **41**: 192-200, 2014b.

Penella C., Nebauer S.G., San Bautista A., López-Galarza S., Calatayud Á.: Improving water stress resistance of pepper through grafting. Abstract in Innovation in vegetable grafting for sustainability. In: 2nd COST action FA1204

514 congress, Carcavelos (Portugal), p. 44, 2014c.

515 Penella C., Nebauer S.G., Lopéz-Galarza S., SanBautista A., Gorbe E.,
516 Calatayud A.: Evaluation for salt stress tolerance of pepper genotypes to be
517 used as rootstocks. – J. Food, Agric. Environ. **11**: 1101-1107, 2013.

518 Pina A., Errea P., Martens H.J.: Graft union formation and cell-to-cell
519 communication via plasmodesmata in compatible and incompatible stem unions
520 of Prunus spp. – Sci. Hortic. **143**: 144-150, 2012.

521 Pina A., Errea P., Schulz A., Martens H.J.: Cell-to-cell transport through
522 plasmodesmata in tree callus cultures. – Tree Physiol. **29**: 809-818, 2009.

523 Pina A., Errea P.: A review of new advances in mechanism of graft compatibility-
524 incompatibility. – Sci. Hortic. **106**: 1-11, 2005.

525 Quilliam R.S., Swarbrick P.J., Scholes J.D., Rolfe S.: Imaging photosynthesis in
526 wounded leaves of *Arabidopsis thaliana*. – J. Exp. Bot. **57**: 55-69, 2006.

527 Rolfe S.A., Scholes J.D.: Chlorophyll fluorescence imaging of plant-pathogen
528 interactions. – Protoplasma. **247**: 163-75, 2010.

529 Rouphael Y., Schwarz D., Krumbein A., Colla G.: Impact of grafting on product
530 quality of fruit vegetables. – Sci. Hortic. **127**: 172-179, 2010.

531 Ruiz J.M., Belakbir A., López-Cantarero I., Romero L.: Leaf-macronutrient content
532 and yield in grafted melon plants. A model to evaluate the influence of rootstock
533 genotype. – Sci. Hortic. **71**: 227-234, 1997.

534 Sánchez-Rodríguez E., Romero L., Ruiz J.M.: Role of Grafting in Resistance to
535 Water Stress in Tomato Plants: Ammonia Production and Assimilation. – J. Plant
536 Growth Regul. **32**: 831-842, 2013.

537 Savvas D., Colla G., Rouphael Y., Schwarz D.: Amelioration of heavy metal and

nutrient stress in fruit vegetables by grafting. – Sci. Hortic. **127**: 156-161, 2010.

Schöning U., Kollmann R.: Phloem translocation in regenerating in vitro-heterografts of different compatibility. – J. Exp. Bot. **48**: 289-295, 1997.

Schreiber U., Schliwa U., Bilger W.: Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. – Photos. Res. **10**: 51-62, 1986.

Tadeo F.R., Gómez-Cadenas A., Ben-Cheikh W., Primo-Millo E., Talón M.: Gibberellin-ethylene interaction controls radial expansion in citrus roots. – Planta. **202**: 370-378, 1997.

Trinchera A., Pandozy G., Rinaldi S., Crinò P., Temperini O., Rea E.: Graft union formation in artichoke grafting onto wild and cultivated cardoon: an anatomical study. – J. Plant Physiol. **170**: 1569-78, 2013.

Wang Y., Kollmann R.: Vascular Differentiation in the Graft Union of in-vitro Grafts with Different Compatibility. Structural and Functional Aspects. – J. Plant Physiol. **147**: 521-533, 1996.

Yin H., Yan B., Sun J., Jia P., Zhang Z., Yan X., Chai J., Ren Z., Zheng G., Liu H.: Graft-union development: a delicate process that involves cell-cell communication between scion and stock for local auxin accumulation. – J. Exp. Bot. **63**: 4219-4232, 2012.

561 **Figure Legends**

562 **Fig 1.** Transversal sections of different graft combinations (see codes in table insert
563 in materials and methods) 30 days after grafting. *A* – A/A, *B* – EGG/ST, *C* – A/A25, *D*
564 – A/C12, *E* – A/B14, *F* – TOM/ST, *G* – A/A5, asterisks (*) show limited fusion
565 between both graft partners, *H* – BEU/A, asterisks represent phenols stained green
566 into the vacuoles, *I* – A/ST. Bars= 200 µm (*A*, *F*, *G*) and 400 µm (*B*, *C*, *D*, *E*, *H* and *I*).
567 Abbreviations: AFS – air filled space; CC – cluster of callus cells; NL – necrotic layer;
568 P – pith cells; Sc – Scion; St – stock; T – traqueid elements; VUF – vascular union
569 formation.

570

571 **Fig 2.** Chlorophyll fluorescence images of F_v/F_m after dark-adapted 30 days after
572 grafting in different plant combinations (see codes in table 1): A/A, A/A25, A/B14,
573 A/C12, A/A5, EGG/ST, TOM/ST, A/ST and A/BEU. The false colour code depicted at
574 the bottom of each image ranges from 0.000 – black to 1.000 – pink. Images were
575 taken from a single representative plant.

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