The effect of C_2-chlorohydrocarbons and their phytotoxic metabolites on photosynthesis of crop plants and natural vegetation

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Abbreviations:

Φ	Yield
A	CO_2 assimilation rate at atmospheric CO_2 concentration
A_{max}	Maximum CO_2 assimilation rate at saturating CO_2 concentration
ABS/CS_M	Phenomenological energy flux (per excited cross section) for absorption
ABS/RC	Specific energy flux (per PSII reaction centre) for absorption
C_a	Atmospheric CO_2 concentration
C_i	Intercellular CO_2 concentration
CE	Carboxylation efficiency
CS	Cross-section
DF	Total driving force for photosynthesis
E	Transpiration
ET_0/CS_M	Phenomenological energy flux (per excited cross section) for electron transport
ET_0/RC	Specific energy flux (per PSII reaction centre) for electron transport
G_t	Total conductance, i.e. boundary layer and stomatal
G_s	Stomatal conductance
k_F	Fluorescence emission
k_H	Heat dissipation
k_N	Nonphotochemical rate constant
k_P	Photochemical rate constant
k_X	Energy migration to PSII
ℓ	Relative stomatal limitation of photosynthesis
PEA	Plant Efficiency Analyser
PEG	Polyethylene glycol
PI_{ABS}	Performance index expressed on absorption basis
PMSF	Phenylmethylsulfonyl fluoride
PSI	Photosystem I
PSII	Photosystem II
φ_{E0}	Quantum yield of electron transport
φ_{P0}	Maximum yield of primary photochemistry
Ψ_0	Efficiency with which a trapped exciton can move an electron further than Q_A^- into the electron transport chain
Γ	CO_2 compensation concentration
Q_A	Primary quinone acceptor
RC	Reaction centres
RC/ABS	Density of active PSII reaction centres per total absorption
RC/CS_M	Density of active PSII reaction centres per excited cross section
Rubisco	Ribulose-1,5-bisphosphate carboxylase-oxygenase

RuBP	Ribulose-1,5-bisphosphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
TECE	Tetrachloroethylene
TR_0/CS_M	Phenomenological energy flux (per excited cross section) for trapping
TR_0/RC	Specific energy flux (per PSII reaction centre) for trapping

Abstract:

The effect of C_2-chlorohydrocarbons and their phytotoxic metabolites on photosynthesis of crop plants and natural vegetation:

The emission of the highly volatile C_2-chlorohydrocarbons tetrachloroethylene (TECE) and 1,1,1-trichloroethane (TCE) into the atmosphere, and their subsequent photochemical or biological degradation to trichloroacetic acid (TCA), proceeds predominantly in industrialised countries. Due to continued industrialisation, increasing use of TECE and TCE as solvent and cleaning agent in the metal and textile industry leads to the build-up of these chloroorganic substances in the atmosphere. Increased urban activities and veldfire catastrophes increase the oxidation potential of the atmosphere which leads to an increase in atmogenically produced TCA in the regions on the lee side of the emitting source.

The uptake of TECE into the plant mesophyll may proceed directly via the stomata or after accumulation in the cuticle, leading to inhibition of growth. On the other hand, TCA can enter the plant either through the stomata, or via the root system after deposition from the atmosphere onto the ground and dissolving in ground- or rainwater. The vegetation of semi-arid and arid zones are affected in particular. As formation of phytotoxic TCA poses a serious threat to sensitive ecosystems such as the grasslands of all continents, determination of its effect is of great scientific significance.

The present study falls within the scope of the research project on the Scientific and Technical Cooperation agreement between South Africa and Germany on the ecotoxicological effect of C_2-chlorohydrocarbons and the deposition of their phytotoxic metabolites on the vegetation around the important industrial region of Johannesburg (EVA-project).

Crop plants, representing C_3 and C_4 crop plants, namely *Phaseolus vulgaris* and *Zea mays* respectively, were grown under controlled conditions and exposed to a range of TCA concentrations, as well as to TECE vapour. The effect of these treatments on the photosynthetic metabolism of the plants were determined using the following techniques:

- Fast phase kinetics of chlorophyll a fluorescence for assessing the effects on the primary processes of photochemistry and assessing plant vitality;
- Infra red gas analysis to determine the effects on photosynthetic gas exchange by quantifying the important parameters of CO_2 assimilation; and
- *In vivo* assessment of the activity of the key-enzyme for CO_2 fixation, Rubisco, including Western-blot analysis.

It could be shown that the sensitivity of *P. vulgaris* (C_3) and *Z. mays* (C_4) towards TCA and TECE differed. The physiological and biochemical effect on these plants also differ. It is postulated that the difference in response is due to the large morphological and biochemical differences between C_3 and C_4 plants.

Low concentrations of TCA had a stimulatory effect on the growth and metabolism of *P. vulgaris*, but inhibition occurred at higher concentrations as a result of limitation of mesophyll processes, i.e. primary photochemistry and/or enzymatic reactions, as was deduced from $A:C_i$ response curves, the JIP-test and determination of Rubisco-activity. No initial stimulation occurred in *Z. mays*, and inhibition was evident even at low levels of TCA exposure, as a result of mesophyll limitation.

In *P. vulgaris* treatment with TECE vapour was responsible for inhibition of mesophyll processes. Marked decreases were evident in efficiency of CO_2 fixation, as shown by the decrease in initial slope of $A:C_i$ response curves. However, the overall vitality of the plant was not markedly affected, according to the values of PI_{ABS} obtained by the JIP-test. *Z. mays* seemed to be more resistant to TECE, showing no marked effects due to the treatment. This was probably because of its specialised anatomy and CO_2 concentrating mechanism.

Two year old pine needles were utilised as bioindicator to assess the occurrence of TCA in natural vegetation over presumed pollution gradients in South Africa. The vitality of the needles was determined in parallel by means of fast fluorescence kinetics and the JIP-test to assess the effect of TCA. A direct correlation existed between TCA content and vitality of the needles. For the transect stretching from the highly industrialised areas of Gauteng in South Africa northwest through Upington to the coast of Namibia, no clear relationship between vitality and TCA content existed, which was probably due to the variation in environmental factors. A direct correlation between the TCA content and plant vitality could be established for the transect from Potchefstroom to Sasolburg. This indicated that TCA could be responsible for the decrease in vitality of natural vegetation. Also it was clear that the levels of TCA in plants near highly industrialised areas like Sasolburg are much higher than in rural areas.

C_2-chlorohydrocarbons like TECE and compounds like TCA, have very definite effects on the photosynthetic metabolism of both crop plants and natural vegetation, and when these effects act in combination with additional environmental factors like drought, it could lead to severe damage and possible desertification in sensitive ecosystems like the grasslands of the world.

Keywords: *Air pollution, bioindicator, C_2-chlorohydrocarbons, crop plants, tetrachloroethene, trichloroacetic acid.*

Opsomming:

Die effek van C_2-chloorkoolwaterstowwe en hul fitotoksiese metaboliete op fotosintese van landbougewasse en natuurlike plantegroei:

Vrystelling van die vlugtige C_2-chloorkoolwaterstowwe tetrachlooreteen (TECE) en 1,1,1-trichlooretaan (TCE) in die atmosfeer, en die daaropvolgende fotochemiese of biologiese omskakeling na trichloorasynsuur (TCA), geskied hoofsaaklik in lande met industriële ontwikkeling. As gevolg van toenemende industrialisering, lei die toenemende gebruik van TECE en TCE as oplosmiddels en reinigers in die metaal- en tekstiel industrieë tot die vermeerdering van hierdie organiese chloorverbindings in die atmosfeer van die suidelike hemisfeer. Toenemende stedelike aktiwiteite en veldbrand-voorvalle verhoog die oksidasiepotensiaal van die atmosfeer, wat lei tot 'n toename in die vorming van TCA van atmogeniese oorsprong aan die ly-kant van die oorsprong van vrystelling.

Die opname van TECE deur die mesofil van plante kan geskied via die stomas, of na akkumulasie in die kutikula, wat lei tot inhibisie van groei. Aan die ander kant kan TCA die plant binnegaan deur die stomas of via die wortelstelsel na neerslag daarvan uit die atmosfeer op die grond en oplossing in grond- of reënwater. Plantegroei van semi-ariede en ariede gebiede word veral geaffekteer. Aangesien die vorming van TCA 'n ernstige bedreiging vir sensitiewe ekosisteme soos die graslande van alle kontinente inhou, is bepaling van die effekte daarvan van groot wetenskaplike belang.

Die huidige studie val binne die fokus van die navorsingsprojek van die Wetenskaplike en Tegnologiese Samewerkingsooreenkoms tussen Suid-Afrika en Duitsland op die ekotoksikologiese effek van C_2-chloorkoolwaterstowwe en die neerslag van die fitotoksiese metaboliete op die plantegroei rondom die belangrike industriële gebiede van Johannesburg (EVA-projek).

Landbougewasse naamlik C_3- en C_4-plante (*Phaseolus vulgaris* en *Zea mays*) is onder gekontroleerde toestande verbou en blootgestel aan 'n reeks van TCA-konsentrasies, asook TECE-dampe. Die effek van hierdie behandelings op die fotosintetiese metabolisme van die plante is bepaal deur gebruik te maak van die volgende tegnieke:

- Vinnige fase kinetika van chlorofil a fluoressensie vir die bepaling van die effek op die primêre prosesse van fotosintese en bepaling van plantvitaliteit;
- Infrarooigasanalise vir die bepaling van die effek op fotosintetiese gaswiselling deur kwantifisering van belangrike parameters van CO_2-assimilering; en
- *In vivo* bepaling van die aktiwiteit van die belangrikste ensieme van CO_2-fiksering, naamlik Rubisco, asook Western-blot analises.

'n Aansienlike verskil vir sensitiwiteit ten op sigte van TCA en TECE was duidelik in *P. vulgaris* (C_3) en *Z. mays* (C_4). Die fisiologiese en biochemiese effek op hierdie plante het ook verskil. Die morfologiese en biochemiese verskille tussen die C_3- en C_4-plante is die moontlike oorsaak vir die verskil in respons.

Lae TCA-konsentrasies het 'n stimulerende effek op die groei en metabolisme van *P. vulgaris* tot gevolg gehad, maar hoër konsentrasies het inhibisie veroorsaak as gevolg van 'n beperking in mesofil-prosesse, i.e. primêre fotochemie en of ensiematiese reaksies, soos afgelei uit A:C_i-responskrommes, die JIP-toets en bepaling van Rubisco-aktiwiteit. Geen aanvanklike stimulasie was sigbaar by *Z. mays* nie, maar inhibisie, selfs by lae konsentrasies van TCA-blootstelling, het voorgekom as gevolg van mesofilbeperking.

In die geval van *P. vulgaris* wat behandel is met TECE-dampe het dit die inhibisie van mesofilprosesse tot gevolg gehad, soos weerspieël deur A:C_i-responskrommes. Opmerklike verskille in die effektiwiteit van CO_2-fiksering was sigbaar, alhoewel die algehele vitaliteit van die plant nie merkbaar beïnvloed is volgens die PI_{ABS} verkry deur die JIP-toets nie. *Z. mays* toon meer weerstand teen TECE, met geen opmerklike effekte as gevolg van die behandeling nie. Die moontlike oorsaak hiervan is die gespesialiseerde anatomie en die CO_2-konsentreringsmeganisme.

Twee-jaar-oue dennenaalde is gebruik as bio-indikator om die effek van TCA op die natuurlike plantegroei te bepaal oor moontlike besoedelingsgradiënte in Suid-Afrika. Die vitaliteit van die naalde is in paralles bepaal met behulp van vinnige fase kinetika van fluoressensie en die JIP-toets. 'n Direkte korrelasie bestaan tussen TCA-inhoud en vitaliteit van die naalde. Vir die transek wat gestrek het vanaf die industriële gebiede in Gauteng in Suid-Afrika noordwes via Upington na die kus van Namibië, was daar nie 'n duidelike verwantskap tussen vitaliteit en TCA-inhoud nie, wat moontlik toegeskryf kan word aan die variasie in omgewingsfaktore. 'n Direkte korrelasie bestaan tussen die TCA-inhoud en vitaliteit van naalde versamel op die transek van Potchefstroom na Sasolburg. Dus dui dit aan dat TCA verantwoordelik is vir die afname in vitaliteit van natuurlike plantegroei, en dat die vlakke van TCA in plante hoër is by industriële gebiede soos Sasolburg, as by landelike gebiede.

C_2-chloorkoolwaterstowwe, soos byvoorbeeld TECE, en verbindings soos TCA het 'n definitiewe effek op die fotosintetiese metabolisme van beide landbougewasse en natuurlike plantegroei, en wanneer die effekte in kombinasie optree met omgewingsfaktore soos droogte, kan dit lei tot 'n ernstige skade en moontlike verwoestyning van sensitiewe ekosisteme soos graslande.

Sleutelwoorde: *C_2-chloorkoolwaterstowwe, bio-indikator, landbougewasse, lugbesoedeling, tetrachlooreteen, trichloorasynsuur.*

Chapter 1: Introduction:

1.1. Literature Review:

1.1.1. Air pollution:

Air is polluted when its natural uses are impaired (Treshow and Anderson, 1989). Since a long time air pollution has influenced plants and vegetation adversely, because air pollutants from natural and man-made sources have been distributed over parts of the biosphere for millennia. Volcanic eruptions, natural forest fires and lightning for example have influenced the vegetations of the earth already during the prehistorical period. During the Middle Ages other extra sources of air pollution were active, for example the primitive, industrial activities in the cities, war fare and city fires. But just in the period of the first industrial revolution from ca 1850 on, antropogenic air pollution became a real threat for all living creatures, especially the more sensitive plants in the surroundings of the local sources (Stöckhardt, 1950).

The damages caused by air pollution in Europe are large and it is generally accepted that there is an urgent need for reduced emissions into the atmosphere. The damages are caused by high ambient air concentrations and depositions of many chemical components, photochemical components, particulate matter and toxic compounds. The concentrations and depositions are dependant on the total mass of pollutants emitted to the atmosphere and its spatial and temporal distribution, transport and transformation processes in the atmosphere, and deposition processes (Moussiopoules, 1996).

Other than the problems of global warming and stratospheric ozone depletion, air pollution problems occur in the troposphere. Pollutants in the troposphere, whether produced naturally (such as terpenes in pine forests) or emitted from human activities (such as smoke from power plants) are moved by air currents, which we commonly call wind. (Vesilind, 1997). Theoretically, contaminated air could become globally dispersed, but dilution from broad dispersal, trapping of pollutants by larger particles, falling out in precipitation and diffusion into the ground and vegetation limit the spread (Treshow and Anderson, 1989). The amount of dispersion is directly related to the stability of the air, or how much vertical air movement is taking place (Vesilind, 1997). Ultimately, pollutant concentrations become reduced to non-harmful levels, often within the first hundred kilometers from their origin (Treshow and Anderson, 1989).

The existence of a threshold in health effects of pollutants has been debated for many years. There are several dose-response curves possible for a dose of a specific pollutant and the response. One possibility is that there will be no effect on human metabolism until a critical

Figure 1.1 Human activities, like industrial development, are a major source of the large variety of pollutants being released into the atmosphere on a daily basis.

concentration (the threshold) is reached. However, some pollutants can produce a detectable response for any finite concentration. Nor do these curves have to be linear. In air pollution, the most likely dose-response relationship for many pollutants is non-linear, without an identifiable threshold but also a minimal response up to a higher concentration, at which point the response becomes severe. The problem is that for most pollutants the shapes of these curves are unknown (Vesilind, 1997).

Whereas non-volatile and low-volatile pollutants such as heavy metals and various high-boiling organic compounds are subject to short-range transport and deposition at local to regional sinks, highly volatile organic substances may undergo supraregional to global dispersion through the air. During air transportation, these substances are exposed to various oxidative, photolytic and hydrolytic processes, which lead to modifications of the chemical structure and associated toxicity of the compounds. As a consequence, secondary pollutants with lower or higher phytotoxicity, may be formed. The direction and rate of the chemical processes occurring in the atmosphere are affected by altitude and climatic factors such as the intensity of various types of water, particulate matter, ozone and hydroxyl radicals in the troposhere. Moreover, geographical and meteorological conditions play a crucial role in the spread of pollutants in the biosphere (Weissflog *et al.*, 1999). Sub-continental-scale circulation is one such occurrence. The vertical transport of aerosols between the surface and the troposhere is controlled by stable discontinuities (Held *et al.*, 1996).

1.1.2. Classification of pollutants:

An important approach to classification of air pollutants is that of primary and secondary pollutants. A primary pollutant is defined as one that is emitted as such to the atmosphere, whereas secondary pollutants are actually produced in the atmosphere by chemical reactions (Vesilind, 1997).

Particulate pollutants can be classified by several means and include dust, fumes, mist, smoke and spray. In the context of air pollution control, gaseous pollutants include substances that are gases at normal temperature and pressure as well as vapours of substances that are liquid or solid at normal temperature and pressure. Among the gaseous pollutants of greatest importance in terms of present knowledge are carbon monoxide, hydrogen sulfide, nitrogen oxides, ozone and other oxidants, and sulfur oxides. Carbon dioxide should be added to this list because of its potential effect on climate. Pollutant concentrations are commonly expressed as micrograms per cubic meter ($\mu g/m^3$) (Vesilind, 1997).

Unwanted constituents of air, or air pollution, can have a detrimental effect on human health, on the health of other creatures, on the value of property, and on the quality of life. Some of the pollutants of human health concern are formed and emitted through natural processes. Gaseous pollutants from natural sources include hydrocarbons in the form of terpenes. Examples of terpenes released by natural vegetation include isoprene from oak trees and monoterpenes from pine trees (Cordova and Merritt, 1999). Man-made sources of pollutants can be conveniently classified as stationary combustion, transportation, industrial process, and solid-waste disposal sources. The principal pollutant emissions from stationary combustion processes are particulate pollutants, such as fly ash, smoke, and sulfur and nitrogen oxides (Vesilind, 1997).

1.1.3. Uptake of air pollutants by vegetation:

The environment is affected directly by air pollutants through deposition, whether it be wet or dry, to exposed surfaces, and indirectly through the effect that trace gases and aerosols have on the global climate (Houghton *et al.*, 1996).

Before physiological or biochemical responses in plants can occur, pollutants must be transferred from the atmosphere to the surface, either in the gas-phase or particle-phase. Deposition of particles on plants depends strongly on particle size and density, wind speed and surface shape and structure (Chamberlain, 1975; Little and Whiffen, 1977). Liquid droplets are more effectively captured than dry particles of the same size (Chamberlain, 1975).

Uptake of air pollutants from the atmosphere depends on more than ambient concentrations. Conductance through the stoma, regulating the passage of ambient air into the cells, is especially critical. Such movement depends on the concentration gradient between the ambient air and the sorptive sites within the leaf. Pollutant flow may be restricted by the physical structures of the leaf or scavenging by competing chemical reactions. Since these conditions may change during exposure, the ambient dose to which the plant is exposed does not necessarily reflect the actual cellular exposure (Guderian *et al.*, 1985).

The initial flux of gases to the leaf surface is controlled by the boundary layer resistance. This is a function of the leaf orientation and morphology, including epidermal characteristics, as well as air movement across the leaf. Thus, more pollutant would enter a leaf when there is some air movement (Guderian *et al.*, 1985; Tingey and Taylor, 1982).

Of the morphological components influencing uptake, pubescence is important since the leaf hairs provide a major, yet relatively inert, area of impact. Cuticular waxes also are important in limiting uptake, even through a thin cuticle (Bennett *et al.*, 1973).

Stomatal resistance, on the other hand is critical. The conductance is determined by stomatal number, size, anatomical characteristics such as the degree to which they may be 'sunken' into the leaf, and the size of the stomatal aperture. When closed there is little or no uptake. Opening is regulated by internal CO_2 content, hydration of the guard cells, temperature, humidity, light, water availability and nutrient status, most notably potassium. It is the osmotic gradient produced by the potassium ions in the guard cells that regulates the guard cell turgor and opening of the stoma (Tingey and Taylor, 1982). Responses of stomata to pollutants may influence further uptake of a gas, loss of water and CO_2 exchange (Unsworth, 1982). After uptake through the stomata, pollutants may be translocated through the plant by the phloem (Simonich and Hites, 1995).

Movement of a pollutant in the liquid phase from the substomatal regions to the cellular sites of perturbation must also be considered to be part of uptake. A pollutant encounters many obstacles along this intercellular pathway. Scavenger reactions, such as with ascorbate, may absorb or neutralise a pollutant, or it may react to form other toxic substances. Also, free radicals may be increased indirectly or by decomposition in solution (Tingey and Taylor, 1982).

Pollutants may also enter the plant by partitioning from contaminated soil to the roots and be translocated in the plant by the xylem (Simonich and Hites, 1995).

1.1.4. Effect of air pollution on vegetation:

The effects of air pollution can be subtle for humans and plants alike. A gradual decline and ultimate death of whole forests can result from air pollution, but the slow insidious demise of forests may escape notice because it can take years. Loss in crop production is even more difficult to quantify (Treshow and Anderson, 1989).

Air pollutants differ in phytotoxicity, but may all be involved in the clear-cut visible injury of plants that are sensitive to them. Symptoms of the effects, especially on the leaves and flowers, are morphological disturbances like curvatures and curlings, but also chlorotic and necrotic injuries of the leaves in different forms, and leaf, flower or fruit abortion, dependent on the affecting components and the plant species and variety (Jacobson & Hill, 1970). Visible damage of air pollution to plants could include white to colored markings on leaves and fruit, or burnt, dry necrotic lesions that eventually drop out of living plant tissues, leaving ragged-edged or shot-holed leaves. These are the obvious symptoms of severe, or acute, exposure to aggressive air pollutants. These are symptoms that are not often seen any more, but which once were common around large industrial centres at the turn of the century and even still today in developing countries. It is the near-background levels of pollutants that are of interest today, i.e. the long-term, low-level or chronic episodes of pollutant exposures (Treshow and Anderson, 1989). Sometimes these symptoms may be rather specific for a certain component, and are used for biomonitoring of the effects of air pollutants (Posthumus,1982).

The basis for all these effects may be found in changes of the plants at the molecular, cellular or higher level. Changes in the outer surface structures (cuticular wax layers for example), in the inner, cellular and other membranes, or in special coding, regulating, or catalysing substances in the plants are the first biochemical lesions, leading to more complicated secondary effects (Koziol & Whatley, 1984). As a result, effects on processes of photosynthesis, respiration, growth, development and reproduction of plants have also been caused by air pollution in practice (Unsworth, 1982).

Specially negative effects on these plant processes may influence the plant performances at the higher levels of organisation, for example in populations, vegetations and ecosystems. Changes in competitive relationships between varieties and species may be the result of the influences of air pollutants, ultimately leading to the disappearance of the most sensitive ones. At extremely high pollution levels the total vegetation and ecosystem may be extinguished (Posthumus, 1991).

Due to the low levels of air pollution in rural areas at greater distances from point sources, one might expect that the selection pressure exerted on plant populations is low. The more sensitive individuals in such a population will be longer retained than in populations exposed

to acute pollutant stress. Yet, plant populations do become tolerant to chronic air pollution. Following alleviation of air pollutant selection pressure, a plant population that has become tolerant to pollution may recover, or become a more sensitive population with a higher degree of variation. The degree of selection pressure will determine the size of the gene pool, making recovery in severely selected populations a slower process than adaptation, since more severe selection will lead to a smaller number of individuals to contribute to the gene pool. Yet recovery can occur within a matter of years, depending on the rate of generative reproduction and differences in the relative fitness of genotypes. Tolerant genotypes are more competitive in the presence of polluted air, but are less fit than sensitive genotypes in clean air, since they have to utilise more of their resources for succesful competition and survival (Dueck, 1991). The influence that air pollutants have on plant populations can also be considerably affected by factors other than the pollutant itself, i.e., abiotic factors such as nutrition, light intensity, humidity and other climatological conditions (Mansfield *et al.*, 1986) or by interactions with biotic factors.

Although effects of air pollutants on plants may not be clear at all levels of organisation, it is evident that the origin of all effects lies at the molecular level, where the reactions with air pollutants take place. Mostly the oxidative properties of the air polluting components or the transformation products of these are responsible for the primary action at the targets. Peroxidation of the fatty acids in membranes, disturbance of disulphide bonds in proteins, oxidation of sulphydryl groups in amino acids etc. are involved in the breakdown of the cell integrity and the inhibition of enzyme activities (Mudd, 1982). Changes in structure and metabolic activities are the result, and ultimately cells may lose their turgor, pigments and physiological functions. In the mechanisms of development in plants, phytohormones can play an important role, although their involvement has not yet been proven undoubtedly. Many processes, which have been shown to be influenced by air pollution, are regulated by phytohormones in general. Disturbances of the hormonal balance may result in several subtle, but also drastic changes in plants (Posthumus, 1991).

As a central theme in air pollution effect studies on plants, the relationships between exposures of the plants and the resulting effects are important for several reasons. Specially the quantitative relationship between the amount of pollutant influencing the plant and the resulting intensity of the effect is equally important. On the basis of these criteria, determined by phytotoxicological research, the national and supranational legislative commissions make their political decisions concerning this matter (Posthumus, 1991).

When several components of air pollution are acting on the vegetation, simultaneously or sequentially, it is very difficult to relate this multiple exposure to the ultimately resulting effects after some time. Another complication of the exposure-effect relationship for effects of air pollutants on plants, is the drastic influence of several environmental conditions and of the

other abiotic and biotic stress factors on the effects. Meteorological conditions, which sometimes cause really strong stresses in the plants, soil and nutrient conditions, and biotic stress factors like pathogens and pests, all may influence the integrated effects of the multi-causal complex. It is clear that the problem of the forest decline is a very good example of this multiple-stress phenomenon (Moseholm *et al.*, 1988).

Most of the research on plants in the past focused on loss of photosynthetically active area due to morphological or physiological injury to leaves and eventually growth reductions. The phases of growth too, are differentially sensitive to air pollutants or pollutant combinations, whether to exposures to chronic levels or to peak concentrations of aerial contaminants. Compared to other, usually persistent pollutants, air pollutants may appear to exert less environmental pressure, especially at chronic concentrations. However, they are clearly strong enough to affect plant processes and to interest scientists in them (Dueck, 1991). In photosynthesis, and the associated biochemical processes, there are numerous steps that could be blocked or interrupted by outside agents such as air pollutants. Not only can the chemistry in the plant cell be disrupted, but the sites of chemical action themselves can be destroyed (Treshow and Anderson, 1989). It is difficult to generalise as to the precise mechanism by which a specific pollutant affects plants, since the precise way in which specific pollutants interfere with normal metabolic processes varies with the pollutant.

The epidermis is the first target of air pollution as the pollutant first passes through the stomata of the epidermal tissue and acts on its opening mechanism. Passing into the intercellular spaces, it dissolves in the surface water of the leaf cells, influencing cellular pH. It may next react with the walls of the mesophyll cells. The pollutant is more likely to react with the cell membrane, notably the protein components, since the cellulose walls are relatively inert. Passing into solution, the pollutant may form free radicals that may be still more reactive and toxic. Pollutants may pass through the outer cytoplasmic cell membrane to come into contact with the cell's organelles, most readily the chloroplast (Treshow and Anderson, 1989).

The inner thylakoid membrane apparently is the most sensitive, with the enzymes of the thylakoid and protein components of the membranes being the most likely targets of pollution. Enzymes essential to CO_2 fixation appear to be especially sensitive (Treshow and Anderson, 1989).

Changes in the ultrastructure of the various organelles provide the first actual symptom of injury. These symptoms, like the foliar symptoms, vary with the pollutant involved. Chlorophyll cannot act in photosynthesis by itself without the membrane structure and ultrastructure provided by intact chloroplasts. Although membranes appear to be most vulnerable, especially the thylakoid which is most vital to photosynthesis, other structures and processes can also be affected. Consequently respiratory, carbohydrate and lipid metabolism, and

practically every other plant process all have been shown to be affected by air pollutants. The net result is an unhealthy plant, at first showing no outward sign of stress. Even before visible symptoms appear, however, the plant is weakened and its growth is impaired. Ultimately, the visible symptoms characterising the presence of a specific pollutant may appear, and death may ensue (Treshow and Anderson, 1989).

The effect of air pollution on seedlings appears to be much stronger than on adult plants. There could be several reasons for this: seedlings are less able to detoxify air pollutants; they are compelled to absorb and take up relatively more pollutant than adult plants; and genetic variation in a seedling population is higher; consequently they are more sensitive individuals (Dueck, 1991).

1.1.5. Effect of specific pollutants:

1.1.5.1. Ozone (O_3):

Air pollutants like ozone, which are photo-oxidants, can cause necrotic lesions on the leaf surfaces of susceptible plants, resulting in economic implications in countries like central Europe and North America where 30% and more of crops are lost due to damage caused by pollutants like ozone (Heagle, 1989; Heck, 1989).

Injuries caused by ozone consist of flecking in broadleaf plants, with total chlorosis occurring in severe cases, and palisade cells being particularly susceptible (Knudson and Tibbitts, 1979). The photosynthetic activity of plants can be inhibited by ozone, due to decreased stomatal conductance or reduction of mesophyll cells' capacity to fix CO_2 (Hill and Littlefield, 1969; Lehnherr *et al*, 1988). Stomata of many species partially close in response to ozone, and since this causes a decrease in the rate of ozone uptake, some species appear to be resistant (Knudson and Tibbitts, 1979). Yield reductions as a result of ozone-exposure can be experienced without visible injury to plants (Heggestadt *et al.*, 1980).

When exposed to ozone, *Triticum aestivum* (wheat) underwent changes in chlorophyll and protein contents and photosynthetic activities, similar to when leafs undergo senescence (Nie *et al.*, 1993). Treatment of *Vicia faba* resulted in a decrease in stomatal conductance, rates of transpiration and photosynthesis measured as CO_2 absorption (Guidi *et al.*, 1993). In *Phaseolus vulgaris* ozone impairs the cells adjacent to the upper leaf surfaces (Leipner *et al.*, 2001).

In rust-susceptible cultivars of *Helianthus annuus* exposed to ozone, fluorescence kinetics revealed immediate closure of reaction centres upon exposure, permament damage as a result of longterm-gassing, a decrease in photochemical quenching, and since the primary electron acceptor Q_A was affected, it implies that photochemistry is impaired (Grobbelaar and Mohn, 2002).

In general, plants respond to ozone by accumulating phenolic compounds, increasing antioxidants and emitting volatile compounds (Schraudner *et al.*, 1997). Also Chappelka and Samuelson (1998) concluded that the effects of ozone on crops are reduction in carbon-fixation, changes in the rates of leaf and root respiration, shifting of the partitioning of carbon into different chemical forms, and disruption of carbon and nutrient allocation patterns.

1.1.5.2. Sulphur dioxide (SO_2):

Effects of sulfur dioxide on plants include bleaching of tissue, as well as more chronic injuries like interveinal chlorosis arising from lysis of chloroplasts in mesophyll cells and the formation of severe areas of white or reddish-brown colouration (Horsman *et al.*, 1979; Ayazloo and Bell, 1981). High concentrations of SO_2 induce stomatal opening, increases in transpiration and inhibition of net photosynthesis (Kondo and Sugahara, 1978).

Decreases in yields of crops exposed to SO_2 occurs even in the absence of visible foliar symptoms (Whitmore and Mansfield, 1983) and although *Phaseolus vulgaris* fumigated with SO_2 showed decreases in yields at lower dosages, higher concentrations caused increases, possibly as a result of a fertilisation effect (Engelbrecht, 1987).

A combined action of SO_2 with H_2S changes the permeability of the limiting plasmalemma towards ions, and even possible structural destruction of organelle membranes and inhibition and decomposition of enzymes, at which stage visible injury of the leave occurs (Hällgren and Huss, 1975).

1.1.5.3. Other pollutants:

Broadleaf species exhibit necrosis after exposure to hydrogen fluoride gas, with net photosynthesis, the Hill reaction in chloroplasts and formation of chlorophyll a and b being inhibited. Binding of Mg^{2+} or divalent heavy metals also causes inhibition of many enzymes (Ballantyne,1972).

Heavy metals like lead inhibit CO_2 fixation, ATP formation and photosystem II activity, and seems to bind to special sites in the cell inducing changes in the permeability of cell membranes (Miles *et al.*, 1972).

1.1.6. C_2-chlorohydrocarbons:

In recent years there has been a growing concern about the possible environmental impact of chlorinated organic compounds. Such compounds are often highly resistant to degradation in nature, which can mean that humans and animals are exposed to the compounds for long periods of time with harmful consequences. Genetic alterations are one such type of possible long-term damage which is important since there is a high degree of correlation between genetic-mutage effects and the development of cancer (Kringstad *et al.*, 1981).

The troposphere contains many species that have the ability to absorb solar radiation and subsequently initiate radical-chain oxidation processes. The 'trigger' of all atmospheric oxidation reactions is considered to be the photolysis of ozone (Seinfeld, 1986). The singlet oxygen atoms produced from photolysis react with water to form hydroxyl radicals. Since these radicals are unreactive towards nitrogen and oxygen they can react with most atmospheric trace species, including highly volatile chemicals such as C_2-chlorohydrocarbons, e.g. tetrachloroethene, to produce trichloroacetic acid (Pienaar and Helas, 1996). C_2-chlorohydrocarbons are subject to increasingly ubiquitous spreading owing to their long-distance transport in the atmosphere. When transported through the air, these compounds are exposed to oxidative, photolytic and hydrolytic processes, which can alter their original structure and thus their chemical and physical properties, as well as their potential phytotoxicity (Weissflog *et al.*, 2001).

In terrestrial ecosystems, plant biomass is believed to play a significant role in the circulation and bioaccumulation phenomena of chlorinated hydrocarbons, and the air to leaf transfer of gaseous organics can be considered a key process, particularly for less soluble compounds. Chlorinated hydrocarbons distribute and cycle between air-water and air-soil by means of deposition-volatilisation periods, with the so-called "grasshopper effect". (Calamari *et al.*, 1991).

When stable and persistent organochlorine compounds was used as insecticides, they caused, and in some cases are still causing, environmental contamination which at least sometimes is of sufficient magnitude to deserve the description of pollution. The danger of organochlorine insecticides is that they may be reconcentrated in living systems. It is suggested that this commonly occurs step by step in food chains, but this process is probably less important than is imagined. It does occur but seldom builds up to anything approaching lethal levels in terrestrial food chains (Mellanby, 1972).

C_2-chlorohydrocarbons can also be formed through iron catalysed reactions of chlorides with humic and fulvic acids (Keppler *et al.*, 2000). Further anthropogenic sources of C_2-chlorohydrocarbons are chemical transformation processes that occur during the burning of coal in large power stations, burning of waste, and waste deposits – especially in special waste depots where the chlorine bleaching of raw cellulose and the chlorination of potable water takes place (Weissflog *et al.*, 2002).

1.1.7. Biomass burning:

Although biomass burnings contribute an uncertain number to overall emissions, it is an important source of trace species and volatile organic compounds to the atmosphere (Helas and Pienaar, 1996a). Today 70-90% of all vegetation fires are believed to be of anthropogenic origin. Biomass burning entails the burning of forests, grasslands and agricultural lands,

following either the harvest for clearing of land or land conversions, or by natural occurrences like lightning. About 2600-4000 x 10^{12} g of biomass is burned annually, with savanna fires seeming to be the single largest source of pyrogenic emissions. Since Africa contains 66% of the world's savanna regions, the fires in African savannas are responsible for about 30% of the biomass burned worldwide (Van Wilgen *et al.*, 1997). The particular species generated by biomass burnings depends on the emission ratio of a particular chemical species, the type of biomass affected, and the burning efficiency of the fire (Helas and Pienaar, 1996b).

Sub-continental-scale recirculation could be responsible for the importation of pollutants emanating from biomass combustion in regions far north of South Africa and together with regional-scale recirculation could play a major role in the transport of airborne pollutants (Tyson, 1997).

Figure 1.2 Biomass burning, and specifically fires in the savanna regions of Africa, is an important source of trace species in the atmosphere.

Biomass burning, and the presence of chloride containing substances like sea water, can lead to C_2-chlorohydrocarbons and directly or indirectly causes the disposition/formation of phytotoxic TCA in plants. In a case study described by Weissflog *et al.* (2002), values for TCA content in pine needles sampled on the Cape Peninsula on 19.05.1999 and 08.04.2000 respectively, rose from 1.6 µg TCA/kg fw to 6.7 µg TCA/kg fw. In the period between sampling the Cape Peninsula was hit by a huge vegetation fire, which was extinguished using seawater. The chlorides in the seawater probably reacted with reactive gases and burning organic substances from the fire to highly volatile chlorohydrocarbons, including TCA (Weissflog *et al.*, 2002).

Thus it can be stated that C_2-chlorohydrocarbons globally produced during biomass burns are converted to the phytotoxic substance TCA after adsorption and that C_2-chlorohydrocarbons are subsequently absorped by the vegetation. These processes may also be applicable for the possible production of other haloacetic acids. One can therefore conclude that these processes of chlorohydrocarbon production due to higher alkaline and earth alkaline halogens in the vegetation and in the upper soil layers may occur more readily during fires in semi-arid and arid zones (Weissflog *et al.*, 2002).

1.1.8. Tetrachloroethene (TECE):

TECE is utilised commercially as a solvent and dry-cleaning liquid. It is colourless, boils at 121°C, and has a specific gravity of 1.60. It is non-inflammable at ordinary temperatures. Physiologically its action is similar to that of carbon tetrachloride (Shepard, 1951).

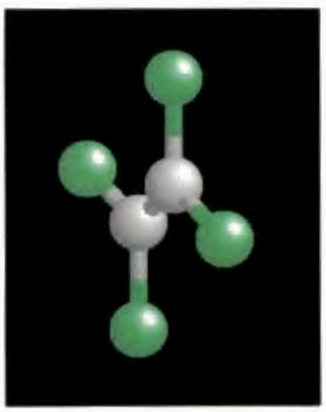

Figure 1.3 Tetrachloroethene (TECE)

About 95% of all TECE produced escapes into the environment, with additional amounts originating from sources such as combustion processes (Weissflog *et al.*, 2001). It has an average lifetime of 84 days in the atmosphere (Franklin, 1994).

Some ethylene derivatives are sufficiently volatile for fumigant applications. Volatility is expressed as the amount that would be present if a unit volume of air were saturated with it at a given temperature and pressure. It is related to the vapour pressure of the liquid. The less toxic a unit of vapour, the more volatile the compound must be to have practical fumigant value. In the ethylene series the toxicity increases with chlorination. The median concentration for TECE is 55mg, and the volatility drops off so rapidly that in practical applications in space fumigation the increased toxicity of the TECE is more than offset by the reduction in volatility (Shepard, 1951).

Ambient air concentrations of TECE are generally below 5 $\mu g/m^3$ in urban areas and typically below 1 $\mu g/m^3$ in rural areas. Indoor concentrations are generally less than 5 $\mu g/m^3$. Indoor TECE air levels may rise to mg/m^3 concentrations in close proximity to dry-cleaning operations where TECE is used as cleaning solvent or in homes where dry-cleaned clothing

is often worn. Inhalation of TECE is the major route of exposure in the general population (Kryzanowski *et al*, 1994).

The main health effects of concern with TECE are cancer and effects on the central nervous system, liver and kidneys. TECE is classified by IARC as a 2A carcinogen (probably carsinogenic to humans). Short-term studies in volunteers (duration 1 or 5 days) showed effects on the central nervous system at >678 mg/m^3. A recent study of dry-cleaning workers with long-term exposure showed that renal effects may develop at lower exposure concentrations, with the reported onset of renal damage occurring after exposure to a median concentration of 102 mg/m^3. Based on the overall health risk evaluation a guideline of 0.25 mg/m^3 is recommended, and it is judged that this guideline value will be protective against less well established effects, including deficits in colour vision (Kryzanowski *et al*, 1994).

1.1.9. Trichloroacetic acid (TCA):

Most herbicides in use today kill plants by a subtle interaction with the physiological or biochemical processes of the plant. They initiate a chain of events that eventually leads to the death of the plant. Such initial actions may involve the inhibition of photosynthesis, the generation or accumulation of toxic molecules, the failure to produce vital intermediary metabolites or the inhibition of plant growth (Hance and Holly, 1963).

TCA was introduced as an organic herbicide in the UK in 1950 (Hance and Holly, 1963). It is a non-selective herbicide (Ashton and Crafts, 1973), and was used in the agriculture against monocotyledonous grasses at an application level of 15-30 kg TCA-Na/ha (Brian, 1976).

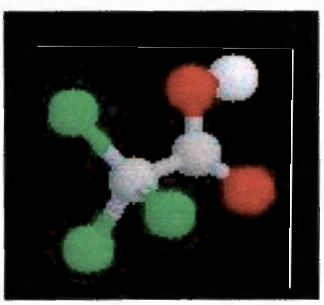

Figure 1.4 Trichloroacetic acid (TCA)

TCA is soil-acting and used pre-planting or pre-emergence. It is readily absorbed by both roots and shoots (Hance and Holly, 1963) and translocated through the entire plants including the leaves, stems and shoot apex, with the largest amounts being found in older leaves. However, small amounts are also transported via the symplast system (Blanchard, 1954). TCA seems to be largely confined to the apoplast, and it is detoxified only slowly, if at all, by the plant (Hance and Holly, 1963). TCA is quite stable in higher plants, and remains undegradated in most species (Blanchard, 1954), with only trichloromethyl compounds being

detected in tomato and tobacco treated with TCA (Mayer, 1957). If not absorbed by plants, TCA can persist in the soil for only a few weeks (Hance and Holly, 1963).

It is assumed that the mode of action of TCA is similar to that of dalapon (Ashton and Crafts, 1973). It causes formative effects (such as increased tillering), growth inhibition, chlorosis, leaf necrosis and contact injury (Hance and Holly, 1963; Mayer, 1957). TCA inhibits growth of both shoots and roots, but at very low concentrations root growth may be stimulated (Mayer, 1957). 8-year-old Scots pine trees and 6-year-old birch trees were harmed by a one-time application of 6.0 g TCA-Na/m^2 of soil, leading to the loss of 2-year-old pine needles and chlorosis in 1-year-old pine needles within 4 months after the treatment was administered, while the birch trees exhibited yellow discolouration of the leaves (Weissflog *et al.*, 2000).

Growth reductions and reduced wax excretions by leaves caused by TCA exposure occur as a result of alterations of cell membranes and changes in permeability (Dewey *et al.*, 1962; Hance and Holly, 1963).

TCA has been shown to affect a range of biochemical processes including carbohydrate, lipid and nitrogen metabolism, but it is likely that these are secondary responses brought about by primary effects on the structures of proteins. Changes in these structures could affect enzyme activity and membrane permeability and so bring about the observed effects (Hance and Holly, 1963).

Effects of TCA on specific plant species include the following: *Elodea* exhibited stem injury after treatment with TCA, with damage to epidermal, cortical and vascular cells, and a 40% reduction in the size of nuclei in cells of the meristem (Gooch and Erbe, 1967); soil-applied TCA increased the reducing-sugar content of wheat seedlings while non-reducing sugar decreased (Rebstock *et al.*, 1953). In leaves of sensitive varieties of *Lupinus lutens* TCA reduced both cuticle and lamina thickness. In resistant varieties the nitrogen metabolism was modified, since an increase in the amount of asparagine and glutamine occurred, as well as a slight increase in protein and β-alanine with no change in the amount of ammonia, while sensitive varieties exhibited a decrease in amides and β-alanine with an increase in free ammonia and protein. Thus TCA acts by inhibiting enzymes involved in conversion of ammonia to amides, thereby allowing accumulation of toxic levels of free ammonia. Furthermore it appears to increase respiration to a degree as well as the amount of bound IAA while decreasing the amount of free IAA, probably as a result of enzyme activation (Treshow and Anderson, 1989).

High solar radiation and low air temperature lead to oxidative stress in plants, increasing the concentrations of active forms of oxygen like OH and O_2H in the chloroplasts. Normally plants can detoxify these toxic compounds. TECE which reach the chloroplasts via the

atmosphere/wax layer/plant cell pathway can be oxidised by OH-, O_2H and Cl-radicals to form TCA in a similar way as in the atmosphere (Figure 1.5). These processes of oxidation thus lead to an increase in the TCA contents of vegetation and ultimately to disturbances of photosynthetic processes. Thus the plant is additionally poisoned by its internal oxidation processes of TECE to form TCA (Weissflog *et al.*, 2001).

In order to withstand attacks by volatile organic compounds, it is essential for plants to possess the ability to detoxify xenobiotics that have already entered the cells. Detoxification can be achieved by enzymatic cleavage or conjugation of the xenobiotics. In principle, the required reaction chain consists of three distinct phases: (I) Oxidation, reduction, hydrolysis; (II) Conjugation; (III) Metabolism/Transport. TCA detected in the leaves of plants may already be a product of a detoxification reaction against volatile chlorohydrocarbons. In this respect the action of P-450 mono-oxygenases has been proposed from results obtained with animals, micro-organisms and single plants. Whilst phase I reactions might lead to the formation of toxic intermediates, plants also possess detoxification enzymes, e.g. glutathione S-transferase. These enzymes are able to form water soluble compounds from xenobiotics by conjugation with the tripeptide glutathione. Conjugates like this can be transported in the plant and are subject to rapid metabolism (Plümacher and Schröder, 1994).

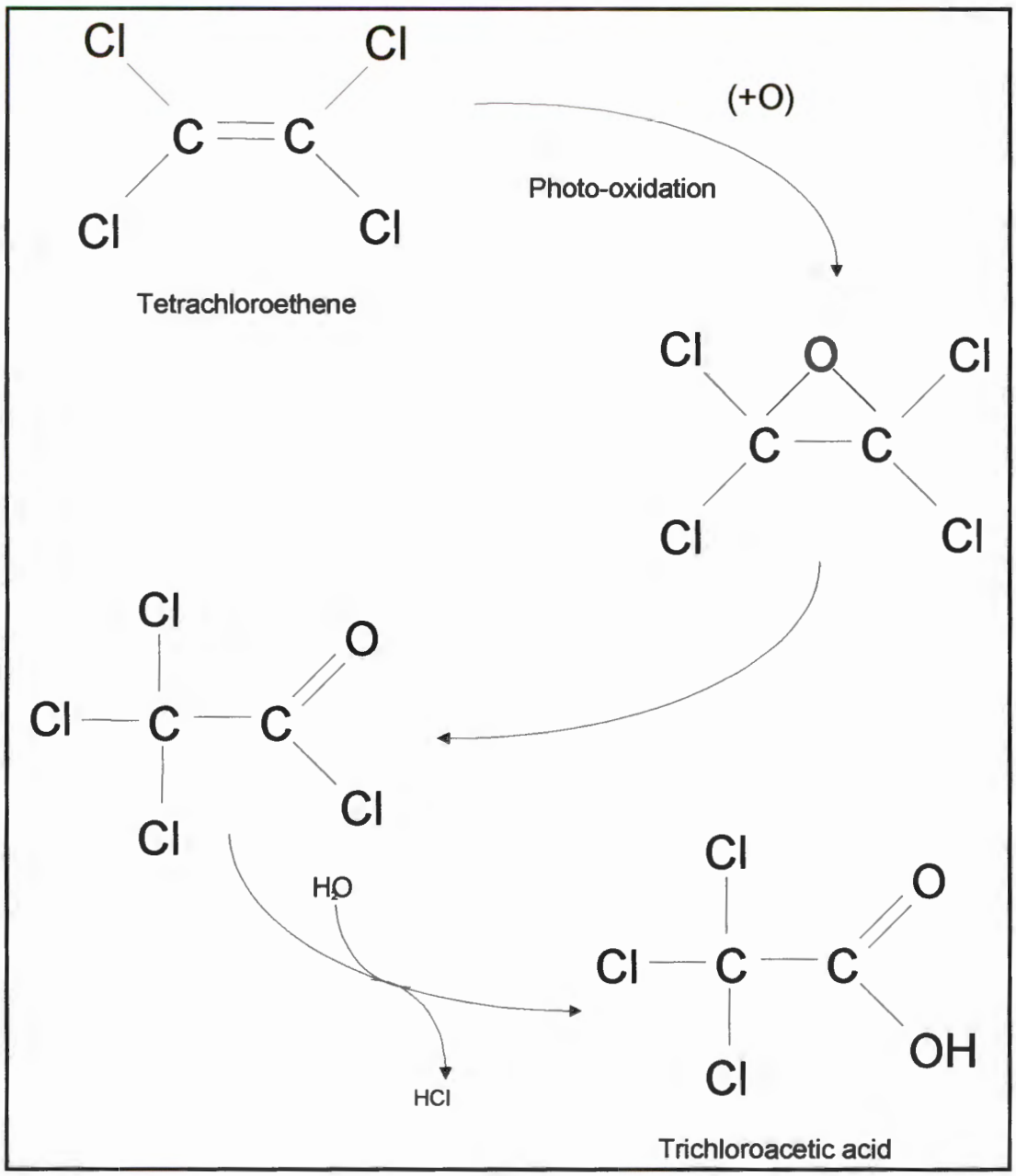

Figure 1.5 Schematic representation of the possible formation of trichloroacetic acid (TCA) from tetrachloroethene (TECE) by photo-oxidation, both in the atmosphere, and by metabolic reactions in plants after uptake through stomata.

1.2 Hypothesis:

Several potential air pollution gradients exist in South Africa, extending from the highly industrialised areas (e.g. Sasolburg/Van der Bijl Park/Johannesburg/Pretoria) to the more remote rural areas. Assessment of the extent of the effect that these pollution gradients have on the environment, and in particular on vegetation, was achieved by utilising pine needles as bioindicator for the levels of certain pollutants in the leaves of plants situated on the gradients.

Experimentation should be able to shed more light on the effect that the C_2-chlorohydrocarbon TECE and its metabolite TCA have on the biochemical metabolism of vegetation, and more specifically on representative C_3- and C_4-crop plants. Theoretically there should be differences in the effect that pollution would have on each type of plant, since important biochemical and anatomical differences between the two exist. It would be expected that the C_4-plant is better suited to deal with the stress of pollution, considering its specialised anatomy and CO_2 concentrating mechanism, which leads to, among other things, a lower stomatal conductance.

1.3 Aim of the study:

The aim of the study was to investigate the effect of TECE and its phytotoxic metabolyte, TCA, on the photosynthetic metabolism of a C_3- and a C_4-crop plant (*Zea mays* and *Phaseolus vulgaris*, respectively).

The investigation was focused on:

- the primary processes of photochemistry, using the fast phase kinetics of chlorophyll a fluorescence to determine the vitality of plants after exposure to the pollutants;
- photosynthetic CO_2 gas exchange, determining the effect of the pollution by quantifying the important parameters of CO_2 fixation and assimilation; and
- activity of the key-enzyme for CO_2 fixation, Rubisco, as well as the effect on protein formation.

Also, the effect of TCA on the vitality of natural vegetation, and specifically *Pinus* spp., over different pollution gradients, was to be investigated, using the pine needles as a bioindicator for the levels of TCA in vegetation, correlating it with the vitality of the plants.

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Chapter 2:
Material and Methods:

2.1. Plants:

Phaseolus vulgaris L. and *Zea mays* L. were used as experimental plants, representing C_3- and C_4- crop plants respectively to study the biochemical effect of TECE and TCA pollution. Seeds of each species were obtained from the Agricultural Research Council, Potchefstroom.

2.2. Growth Conditions:

The experiments were conducted under controlled conditions in growth chambers (Conviron PGW 36, Controlled Environment Ltd, Winnipeg, MB. Canada R3H 0R9) with sufficient incandescent light bulbs (General Electric, Neodynium R80, 100W) and fluorescent lamps (General Electric, Cool White, 1500W) to maintain light intensities as high as 1300 µmole photons.$m^{-2}.s^{-1}$ at 1 m distance. Seeds of each species were planted in pots with measurements of 15 cm x 15 cm x 15 cm containing sterile sand and watered daily with 250 ml of distilled water. The plants were subjected to a daily light period of 15 hours and dark period of 9 hours. During the light period a temperature of 23°C was maintained, and during the night period the temperature was kept constant at 20°C. The CO_2-level was kept constant at the normal atmospheric value of 350 µmoles.$mole^{-1}$ (Figure 2.1).

After a period of two weeks, when the plants had entered the vegetative phase, the number of plants in each pot was reduced to three. 250 ml of complete Hoagland's nutrient solution was administered to each pot every third day onwards. The different treatments were initialised at this point in time.

2.3. Trichloroacetic acid, CCl_3COOH (TCA):

TCA was administered as sodium salt solution at different concentrations to the growth medium (soil) of the test plants. The concentration range used was determined in consultation with Dr. L. Weissflog of the Department of Chemical Ecotoxicology, UFZ Environmental Research Centre, Leipzig-Halle, Leipzig, Germany.

The concentrations applied were 0.05, 0.20, 0.80 and 3.20g TCA per m^2. Investigations conducted by Weissflog *et al.* (2000) employed a concentration of 6.0 g TCA/m^2 in the soil of large perennial trees like pines and birch, with clear visual effects within four months of treatment. Thus lower concentrations were used in these experiments, since the treatments were administered to smaller, annual crop plants. A higher level of 3.20g TCA per m^2 was also used to confirm that very high concentrations have a definite and drastic effect on vegetation. The control pot received no TCA.

(a)

(b)

Figure 2.1 *Zea mays* (a) and *Phaseolus vulgaris* (b) grown under controlled conditions in computerised growth chambers at a light intensity of 1300 µmole photons.m^{-2}.s^{-1} and CO_2-level of 350 µmoles.$mole^{-1}$, with a light-regime of 15 hours at 23ºC, and dark-periods of 9 hours at 20ºC.

The surface area of the soil in the pots was determined (225 cm^2) and used to calculate the TCA dosage for each treatment. The TCA-salt was dissolved in 100ml distilled water before it was applied to the soil. The TCA was administered only once. Three days after the TCA-treatment was applied, the measurements were made.

2.4. Tetrachloroethene, C_2Cl_4, (TECE):

For TECE-treatment the plants were incubated in specially constructed glass chambers having the dimensions of 1.8m x 0.7m x 0.8m (volume = 1.008m^3), at a light intensity of 500 µmole photons.m^{-2}.s^{-1}. Two glass-cases were used: one served as a control chamber to which no TECE was administered, and a second, in which the plants were exposed to TECE. A volume of 500 µl volatile TECE was administered daily to the glass-case in a petri-dish and allowed to evaporate into the air to obtain a final concentration of ±800 µg/m^3. This end TECE concentration in the chamber was also determined in consultation with Dr. L. Weissflog, ecotoxicologist of the UFZ, Leipzig-Halle, Germany. The plants were kept under these conditions for a period of seven days, after which the plants were transferred back to the original growth chambers the night before the day of measuring.

2.5. Chlorophyll-fluorescence measurements:

Chlorophyll fluorescence transients were measured one hour before the light period started, i.e. on dark adapted plants. The measurements were conducted with a Plant Efficiency Analyser (PEA, Hansatech, Kingslynn, UK), which has a high time resolution (10 µs) and a large data acquisition capacity over several orders of magnitude (Strasser *et al.*, 1995). The transients were induced by a homogenous red light (peak at 650 nm) of 600 W.m^{-2} (3200 µmoles photons.m^{-2}.s^{-1}) provided by an array of six light-emitting diodes, and recorded for 1 second (Strasser *et al.*, 1995) on a 4mm diameter area of a leaf sample.

The measurements were done on the youngest vegetative intact leaves of the experimental plants. In total sixty measurements were made for each concentration of TCA/TECE.

2.6. Photosynthetic gas-exchange measurements:

Photosynthetic CO_2-gas exchange was measured with an open system computerised infrared gas analyser (CIRAS-1, PP-Systems, Hertz, UK). A total of four measurements were taken for each concentration of TCA/TECE-treatment, each on a different plant. A 2.5 cm^2 section of the youngest vegetative leaf of a plant was clamped in a computerised broad leaf cuvette with light and temperature control. During each measurement the light intensity was kept at 1200 µmole.m^{-2}.s^{-1}, and the temperature at 23°C. The ambient CO_2-concentration (C_a) was increased with five-minute increments from 0 to 2000 µmole.$mole^{-1}$, allowing generation of CO_2 assimilation rate (A) *vs* intercellular CO_2 concentration (C_i) response curves.

Figure 2.2 Three-week old *Zea mays* plants after one week of exposure to different TCA concentrations. From left to right: Control (no TCA), 0.05, 0.20, 0.80 and 3.20 gTCA/m^2.

Figure 2.3 Three-week old *Phaseolus vulgaris* plants after one week of exposure to TCA of different concentrations. From left to right: Control (no TCA), 0.05, 0.20, 0.80 and 3.20 g TCA/m^2.

Figure 2.4 TECE-treatment was applied in specially constructed glass-chambers which were placed in a controlled growth-chamber with a light-intensity of 500 µmoles photons.$m^{-2}.s^{-1}$.

Figure 2.5 Three-week-old *Zea mays* after one week of exposure to TECE. On the left the control plant not exposed to TECE, and on the right the plant exposed to TECE.

Figure 2.6 Three-week-old *Phaseolus vulgaris* after one week of exposure to TECE. On the left the control plant not exposed to TECE, and on the right the plant exposed to TECE.

Figure 2.7 Automatic cuvette of the infrared gas analyser (CIRAS-1), with controlled light intensity, temperature and CO_2-levels, determining the photosynthetic gas exchange of a 2.5 cm^2 of leaf.

Figure 2.8 Fluorescence measurements were conducted with a Plant Efficiency Analyser (PEA, Hansatech, Kingslynn, UK), working on a 4mm diameter area of a dark adapted leaf sample.

2.7. Determination of Rubisco-activity:

Leaf discs (3.14 cm^2) were sampled with a freeze clamp at the temperature of liquid nitrogen, at the end of each period of treatment for the determination of Rubisco activity. Sampling occurred 5 hours after the light-period started at a light intensity of 1000 µmol $m^{-2}.s^{-1}$ and temperature of 23°C. Special care was taken not to shade the portion of the leaf from which the sample was taken, since it would have caused deactivation of Rubisco. Leaf discs were stored at -84°C.

To assay Rubisco activity, the method of Keys & Parry (1990) was employed. Each leaf disc was ground in liquid nitrogen and insoluble polyvinylpolypyrrolidone (PVPP), and then rapidly extracted with 800 µl extraction buffer (pH 8.0), containing 100 mM Bicine-NaOH, 20 mM $MgCl_2$, 50 mM β-mercaptoethanol and 5 mM phenylmethylsulfonyl fluoride (PMSF) to form a crude extract.

The crude extract was transferred to a pre-cooled micro centrifuge tube and centrifuged at 10000 x g and 4°C for 1 min. Twenty-five microliter of the clarified supernatant was then added to a glass scintillation vial containing 475 µl assay buffer (pH 8.2). The assay buffer contained 100 mM Bicine-NaOH, 20 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$ (0.5 µCi $µmol^{-1}$) and 400 µM ribulose-1,5-bisphosphate (RuBP). This was done to determine the initial activity of the enzyme, which is the activity under growth conditions at the time of sampling. The reaction took place for 1 min after which it was terminated with 200 µl 10 M formic acid.

To determine the maximum activity of the enzyme, which is obtained by activation of the extracted enzyme with bicarbonate after removal of all known tight-binding inhibitors of rubisco, 100 µl of the supernatant was added to a pre-cooled microcentrifuge tube containing 100 µl 600 mM Na_2SO_4. Incubation in the presence of Na_2SO_4 removes all known tight-binding inhibitors from rubisco catalytic sites. After 30 min of incubation, 200 µl 60% (w/v) polyethylene glycol 4000 (PEG 4000) was added and after further incubation for 30 min, the mixture was centrifuged at 10000 x g and 4°C for 10 min. The supernatant containing the unbound inhibitors was discarded and the pellet washed with 500 µl of a 1:1 mixture containing extraction buffer and 60% PEG 4000. After 2 min the mixture was again centrifuged at 10000 x g and 4°C for 10 min. The supernatant was discarded and the pellet resuspended in 100 µl extraction buffer. Twenty-five microliter of the resuspended pellet was added to a glass scintillation vial and rubisco fully activated in the presence of assay buffer and $NaH^{14}CO_3$. After 3 min 400 µM RuBP was added to start the reaction. The reaction was terminated after 1 min with 200 µl 10M formic acid.

Acidified samples were evaporated to dryness in an oven. After addition of 0.4 ml distilled water and 3.5 ml scintillation cocktail (Packard Ultima Gold) to each scintillation vial, the ^{14}C

incorporated into 3-phosphoglycerate was determined by liquid scintillation spectrometry using a liquid scintillation analyser (Beckmann, LS 6000TA).

The soluble protein content of the original supernatants and the resuspended pellets was determined according to the method of Bradford (1976). The chlorophyll content of each leaf disc was determined according to the method of Wintermans & de Mots (1965). Fifty microliter of the crude extract (prior to centrifugation) was added to 950 µl pre-cooled 96% ethanol, centrifuged for 5 min at 10000 x g and 4ºC, and the absorbance of the supernatant measured at 654 nm.

2.8. Detection of proteins by Western blot analysis:

2.8.1. Extraction of proteins:

Six leaf discs from each treatment were ground to a fine powder with liquid nitrogen in a pre-cooled mortar and extracted with 3 ml ice-cold extraction buffer containing 50 mM Tris-HCl, 1 mM EDTA, 3 mM DTT, 6 mM PMSF, and 30 mg insoluble PVPP. The crude extract was transferred to a pre-cooled micro centrifuge tube and centrifuged at 10000 x g at 4°C for 15 min. The soluble protein content of the supernatant was determined according to the method of Bradford (1976).

The supernatant was diluted in ultrapure water to a concentration of 4 µg total soluble protein in a final volume of 50µl. Fifty microliters SDS-PAGE sample buffer containing 125.5 mM Tris-HCl (pH 6.8), 10% (w/v) SDS, 0.287 mM β-mercapto-ethanol, 0.015 mM bromophenol blue and 20% (v/v) glycerol was added and then boiled for 5 min. Ten microliter of SDS-PAGE sample buffer was also added to 10 µl of western blotting molecular weight marker proteins and boiled for 5 min. The molecular weights of these marker proteins were 10, 20, 30, 45, 75 and 100 kDa. The boiled samples and markers proteins were centrifuged at 10 000 x g and 4°C for 5 min and stored at –85°C.

2.8.2. Electrophoresis:

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used for the separation of proteins and protein subunits. In this technique the proteins are denaturated with SDS, β-mercapto-ethanol and boiling.

Proteins were separated by SDS-PAGE with a mini-gel system (Mighty Small II, SE 250, Hoefer Scientific Instruments, San Francisco). The running gel solution contained 12% (w/v) acrylamide, 0.3% (w/v) bisacrylamide and 0.381 M Tris-HCl (pH 8.8) and was degassed prior to casting. The gel was casted between the glass plates after addition of 61 µl 10% (w/v) SDS, 61 µl 10% (w/v) ammonium persulphate and 10 µl TEMED. The stacking gel solution contained 4% (w/v) acrylamide, 0.1% (w/v) bisacrylamide, 0.118 M Tris-HCl (pH 6.8) and was degassed prior to casting. The gel was casted on top of the polymerised running gel after

addition of 40 µl 10% (w/v) SDS, 21 µl 10% (w/v) ammonium persulphate and 20 µl TEMED. A plastic gel comb was immediately inserted into the stacking gel solution to form 10 sample wells in which the protein extractions and marker proteins were loaded. After the stacking gel has polymerised, the gel comb was carefully removed and the sample wells rinsed thoroughly with SDS running buffer consisting of 78 g Glycine, 15.15 g Tris and 5 g SDS in 5 dm^3 distilled water. In sample wells 2 - 8, 10 µl extract (containing 0.4 µg total soluble protein) was loaded with a micro syringe, while 10 µl of the marker proteins were loaded in sample wells 1 and 10. Proteins were separated for 75 min at 200 V and 4°C.

2.8.3. Protein transfer:

After separation with SDS-PAGE, proteins were transferred from the running gel to a PVDF western blotting membrane (pore size of 0.45 µm). The high binding capacity, high mechanical strength and chemical resistance of the PVDF membrane makes it especially useful for protein transfer. Prior to protein transfer, the membrane was moistened with methanol for 3 s and then rinsed in ultra pure water twice for 1 min. The membrane, as well as two double layers of filter paper (Whatmann #1), was soaked in transfer buffer containing 48 mM Tris, 39 mM Glycine, 20% (v/v) methanol and 1.3 mM SDS for 30 min. The running gel was carefully removed from the glass plates and soaked for 1 min in transfer buffer. The membrane was placed on top of a double layer of filter paper on a semi-dry blotter (Trans-Blot SD, Bio-Rad Laboratories). The gel was then placed on top of the membrane and covered with a double layer of filter paper. Care was taken to remove any air bubbles between the different layers. Protein transfer from the gel to the membrane was conducted for 30 min at 15 V.

2.8.4. Binding of primary antibodies, blocking and labeling with secondary antibody:

The PVDF membrane containing the transferred proteins was washed twice for 5 min in TBS [50 mM Tris-HCl (pH 7.5) and 150 mM NaCl]. The membrane was then blocked for non-specific binding of antibodies by incubation (with gentle shaking) in 1% blocking solution [BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit), Roche Diagnostics GmbH, Roche Molecular Biochemicals, Mannheim, Germany] for 1 h. The membrane was then incubated for 1 h in 0.5% blocking solution containing the primary antibody specific to rubisco at a 1 : 1000 dilution. The membrane was washed three times in TBST [50 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.1% (v/v) Tween 20] for 10 min, and twice with 0.5% blocking solution for 10 min. The membrane was then incubated for 30 min with 40 mU ml^{-1} horseradish peroxidase (POD)-labeled secondary antibody and 25 mU ml^{-1} antibody specific to the marker proteins, diluted in 0.5% blocking solution. The membrane was then washed four times with large volumes of TBST for 15 min each time to remove any unbound secondary antibody.

2.8.5. Chemiluminescent detection of labeled proteins:

The whole procedure was conducted in a dark room. Excess buffer was drained off and the membrane placed in a tray with the protein-containing side facing upward. The pre-mixed

detection reagent, luminescence substrate solution and starting solution (BM Chemiluminescence Western Blotting Kit) was added in a ratio of 100:1 and the membrane incubated for 60 s. Excess detection reagent was drained off and the membrane wrapped in Saran wrap. The membrane was inserted, protein-containing side facing upward, into a X-ray film cassette with a sheet of ECL Hyperfilm (High Performance Chemiluminescence Film, Amersham-Pharmacia Biotech, UK) on top off the membrane. The film was exposed for 10 s in the cassette. The film was then soaked in developer reagent (Ilford Phenisol, Ilford Imaging Limited, Mabberley Cheshire, England) for 5 min, in 3% acetic acid (stop reagent) for 1 min and in a standard fixer reagent for 2 min and then allowed to dry.

Data analysis of Western blots was done using a Syngene Genius Bio Imaging System (Syngene, Beacon House, Nutfield Road, Cambridge CB4 1TF, UK). The software used to photograph the Western blots was GeneSnap (version 5.0) and the software used for estimating the protein content on the blots was GeneTools (version 3.00).

2.9. Statistical analysis:

Significant differences between the means of different treatments were determined by analysis of variance, using Student's t-Test of honest significance difference.

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Chapter 3:
Effect of TCA and TECE on C_3- and C_4-crop plants

3.1. CO_2-gas exchange:

3.1.1. CO_2-gas exchange measurements:

Traditional measuring of the growth of plants, in terms of dry weight and carbon gain, consisted of drying, weighing and chemical analysis of the dried material. Direct measuring of CO_2 uptake provides a complementary approach. Harvesting methods are appropriate for assessment of long-term changes, but not suitable when interest lies with short-term carbon gain or the contributions made by individual organs. The advantages of measuring CO_2 uptake is that it is instantaneous and non-destructive, above the fact that it is an alternative and direct method.

To study CO_2 gas exchange in plants, a number of measures, terms and units are applied (Von Caemmerer and Farquhar, 1981): The assimilation rate (A) is expressed as the amount of CO_2 assimilated per unit leaf area and time (µmol CO_2 $m^{-2}.s^{-1}$). The stomatal conductance (G_s) represents the ratio of flux through the stomata to the CO_2 gradient, with the same dimensions as A, since the CO_2 concentration gradient is dimensionless.

When an open infra red gas analysis system like the CIRAS-1 (PP-Systems, Hertz, UK) is used in measuring photosynthetic CO_2 gas exchange, air is pumped from the cuvette enclosing a leaf into an infrared gas analyser which continuously records the CO_2 concentration of the system. The CO_2 concentration of the system will decrease if the leaf contained in the cuvette is photosynthesising. This decrease will be maintained until the CO_2 compensation concentration of photosynthesis is (Γ) reached. The CO_2 assimilation rate equals the change in the amount of CO_2 in the system per unit time. Changes in temperature and pressure are compensated for in the calculation of the assimilation rate, but humidity has to be controlled, since a rise in transpiration will cause an increase in the amount of water vapour, causing dilution of CO_2 as well as the other gases in the system (Long and Hällgren, 1993).

An *in vivo* screening of limitations to the photosynthetic carbon assimilation can be achieved with studies of CO_2 gas exchange. Such studies allow the quantitative assessment of environmental factors on the different steps in the diffusion pathway. It is possible to separate limitations in the mesophyll from stomatal limitations by employing simultaneous measurements of CO_2 and water vapour fluxes. Separation of the effects on the CO_2 limiting

phases of photosynthesis is also possible (Long and Hällgren, 1993; Farquhar and Sharkey, 1982).

CO_2 must first diffuse through the boundary layer before it is able to reach the leaf. The aerodynamic properties of the leaf and leaf chamber, as well as wind speed and turbulence are brought into calculation to determine the boundary layer conductance. The boundary layer conductance will be at least an order of a magnitude greater than the highest possible stomatal conductance value (G_s) under field conditions (Long, 1985). In the Parkinson cuvette the boundary layer resistance is minimised and kept constant by the fan of the cuvette, which keeps the turbulence high.

If only diffusion is taken into consideration, the intercellular CO_2 concentration (C_i) can be determined by:

$$C_i = C_a - A/G_\ell$$

where G_ℓ represents the total conductance, i.e. boundary layer as well as stomatal, and C_a is the atmospheric CO_2 concentration. The amount of gas that leaves a photosynthesising leaf is usually more than the amount that enters it, resulting in a pressure gradient which causes the outflow of CO_2 and other gases. This decreases the amount of C_i relative to C_a. Transpiration (E) should however also be taken into account, since the aforementioned will occur even if no CO_2 is consumed in the mesophyll (Von Caemmerer and Farquhar, 1981), thus leading to:

$$C_i = [(G_\ell - E/2) \cdot C_a - A]/(G_\ell + E/2)$$

3.1.2. A:C_i response curves: Quantification of data:

Plotting the rate of CO_2 assimilation (A) against the intercellular CO_2 concentration (C_i), provides an illustration of the response of A to C_i when no stomatal limitations are present ($C_a = C_i = 350$ $\mu mol.mol^{-1}$ CO_2) (Figure 3.1).

To distinguish between stomatal and mesophyll limitation, the following equation is employed:

$$\ell = (A_0 - A)/A_0$$

where the assimilation rate (A) measured at normal ambient CO_2 concentration (C_a = 350 $\mu mol.mol^{-1}$) is subtracted from the assimilation rate that would occur if no stomatal limitations were present (A_0) (Farquhar and Sharkey, 1982). Stomatal limitation (ℓ) represents the proportionate decrease in CO_2 assimilation that may be attributed to the stomata.

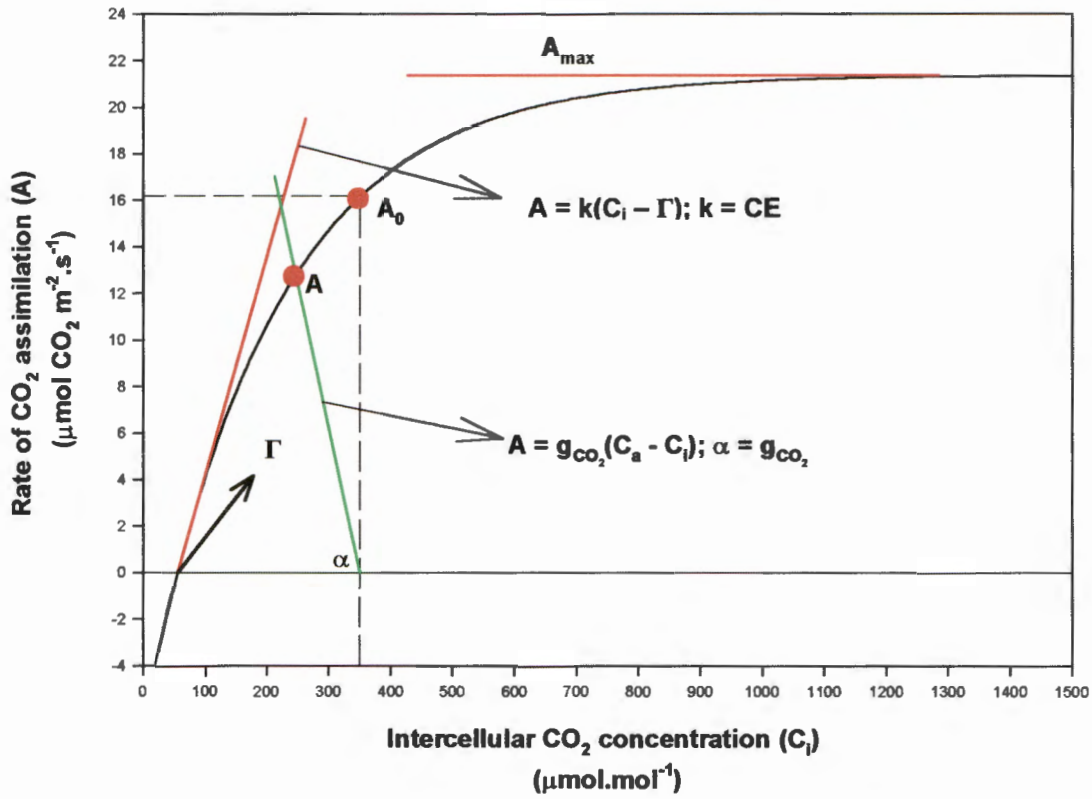


Figure 3.1 Response of CO_2 saturated assimilation rate (A) vs intercellular CO_2 concentration (C_i). A is the CO_2 assimilation rate under the given conditions, i.e. the point of simultaneous solution of the demand and supply functions. Carboxylation efficiency (CE) is represented by the initial slope of the demand function, the CO_2 compensation concentration (Γ) is the intercellular CO_2 level where the net usage of CO_2 equals zero, the maximum rate of assimilation (A_{max}) represents the rate of CO_2 assimilation at saturated levels of CO_2, and A_0 is the rate of assimilation that would occur if no stomatal limitations were present (interpolation of the value of A from the response curve at C_i = 350 µmol.mol^{-1}).

The carboxylation efficiency of photosynthesis can be deduced from the initial linear response, i.e. $\delta A/\delta C_i$, which is an *in vivo* measure of the amount of active ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) (Bolhàr-Nordenkampf and Öquist, 1993).

3.1.3. Results and Discussion:

3.1.3.1. Effect of TCA on photosynthetic CO_2-gas exchange:

3.1.3.1.1. *Phaseolus vulgaris*:

The dependence of the change in the rate of CO_2 assimilation (A) to the change in intercellular CO_2 concentration (C_i) in *Phaseolus vulgaris*, was markedly affected by the TCA treatment (Figure 3.2). Conspicuously, the initial slope (CE) and A_{max} were higher than the control for the two lower TCA treatments. At the higher TCA treatments, however, these parameters were suppressed. The effect of TCA on the different parameters of photosynthetic gas exchange, with indications of statistically significant changes, are presented in Table 3.1.

Upon treatment of *Phaseolus vulgaris* with a range of increasing TCA concentrations, the rate of CO_2 assimilation at a C_a of 350 µmol.mol^{-1} (A_{350}) increased with up to 21% at a TCA level of 0.20 g/m^2, after which a decrease was observed, with A_{350} declining 39% ($p<0.05$) at a treatment of 3.20 g TCA/m^2 (Figure 3.3a). No significant changes occurred in the intercellular CO_2 concentration, despite the corresponding decrease in A_{350}, indicating the decrease in Rubisco activity (which is also reflected by the decrease in CE) at the higher TCA levels (Figure 3.3b). The stomatal conductance at ambient C_a (Figure 3.3c) decreased markedly with increasing TCA concentrations. The maximum rate of CO_2 assimilation (A_{max}) increased with 13% ($p<0.01$) and 15% ($p<0.05$) at the lower TCA concentrations of 0.05 and 0.20 g/m^2 respectively, while a decrease of 5% ($p<0.05$) was observed at the higher dosage of 3.20 g TCA/m^2 (Figure 3.4a). The initial slope of the demand function, i.e. carboxylation efficiency ($\delta A/\delta C_i$), showed an initial, although statistically insignificant, increase of 14% at 0.20 g TCA/m^2, followed by a decrease of 38% ($p<0.01$) at a treatment of 3.20 g TCA/m^2 (Figure 3.4b).

The observation of an initial increase in A_{350}, CE and A_{max}, corresponded with the visual appearance of the plants: The plants treated with 0.05 and 0.20 g TCA/m^2 were conspicuously larger at comparable growth stages. This phenomenon also corresponded to the initial stimulation at lower concentrations of TCA exposure and subsequent decrease at higher levels observed in fluorescence parameters (3.2.2.1.1.) and the activity of Rubisco (3.3.2.2.1.).

The CO_2 compensation concentration (Γ) increased gradually up to 27% ($p<0.01$) at a TCA concentration of 3.20 g/m^2 (Figure 3.4c), pointing at increased mesophyll limitation, while the stomatal limitation of photosynthesis (ℓ) underwent an insignificant increase of 29% at the highest treatment of 3.20 g TCA/m^2 (Figure 3.5), corroborating the above mentioned

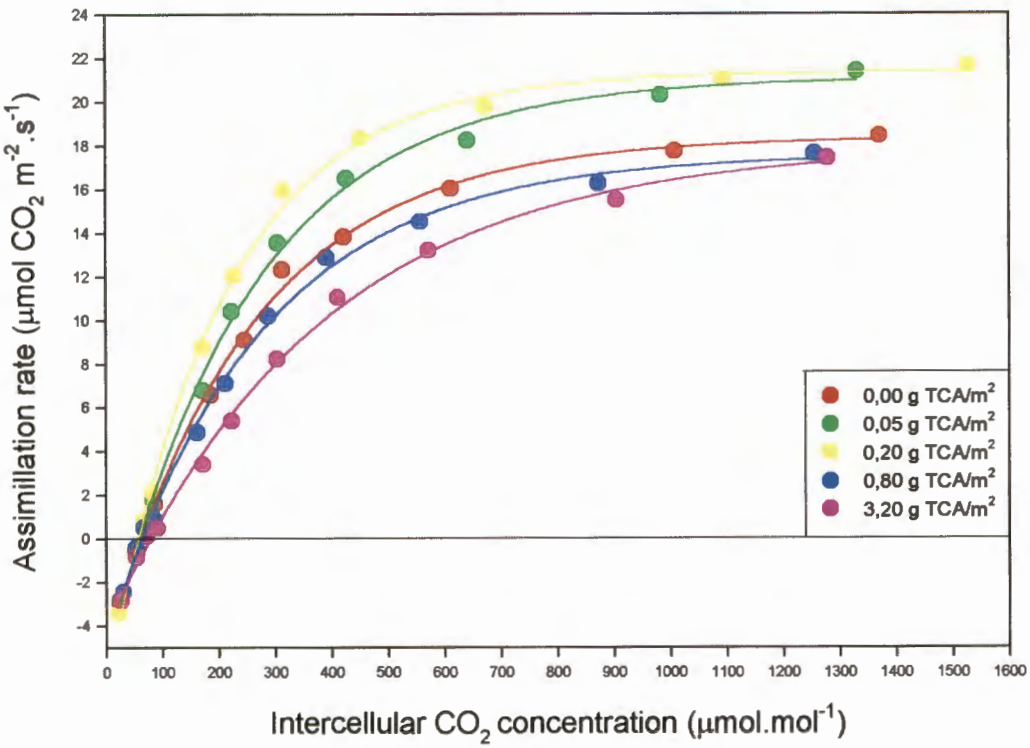
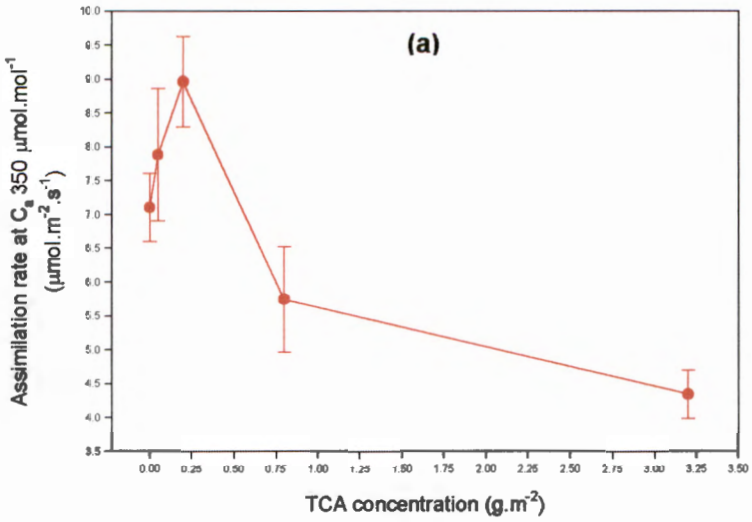
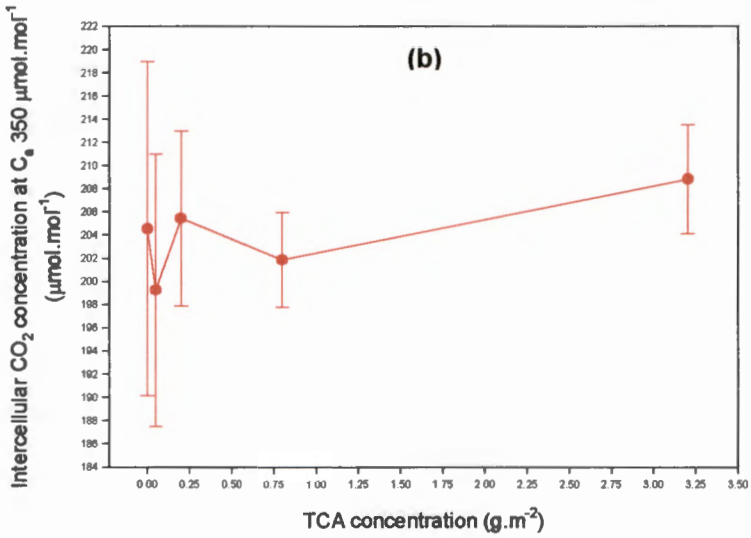


Figure 3.2 CO_2 assimilation rate (A) vs intercellular CO_2 concentration (C_i) response curves for *Phaseolus vulgaris* exposed to TCA concentrations of 0.05, 0.20, 0.80 and 3.20 g/m^2. The plotted values represent the mean of five replicates.

Table 3.1 Rate of CO_2 assimilation (A_{350}) (µmol.$m^{-2}.s^{-1}$) at C_a = 350 µmol.mol^{-1}; intercellular CO_2 concentration (Ci_{350}) (µmol.mol^{-1})at C_a = 350 µmol.mol^{-1}; stomatal conductance (Gs_{350}) (m.s^{-1}) at C_a = 350 µmol.mol^{-1}; maximum rate of CO_2 assimilation (A_{max}) (µmol.$m^{-2}.s^{-1}$); initial slope of the demand function ($\delta A/\delta C_i$) (mol.$m^{-2}.s^{-1}$); CO_2 compensation point (Γ) (µmol.mol^{-1}) and stomatal limitation of photosynthesis (ℓ) (%) for *Phaseolus vulgaris* treated with a range of TCA concentrations, with * and ** indicating significant differences ($p<0.05$ and $p<0.01$ respectively) compared to untreated plants.

	Untreated	0.05 g TCA/m^2	0.20 g TCA/m^2	0.80 g TCA/m^2	3.20 g TCA/m^2
A_{350}	7.1±1.2	7.88±1.3	8.96±0.67	5.74±0.78	4.34±0.36*
Ci_{350}	204.5±14.4	199.2±11.8	205.4±7.6	201.8±4.1	208.8±4.7
Gs_{350}	137±43.9	101.8±28.5	92.5±14.4	67±8.5	53±3.4
A_{max}	17.8±0.62	20.4±0.31**	20.84±0.98*	16.82±1.0	16.84±1.1*
$\delta A/\delta C_i$	0.056±0.0026	0.058±0.0057	0.065±0.002	0.044±.0056	0.035±0.0029**
Γ	58.75±5.91	59.8±2.2	61±2.14	67.6±2.36	80±3.54**
ℓ	35.08±7.11	45.93±4.82	43.3±4.3	45.32±2.62	49.66±4.63





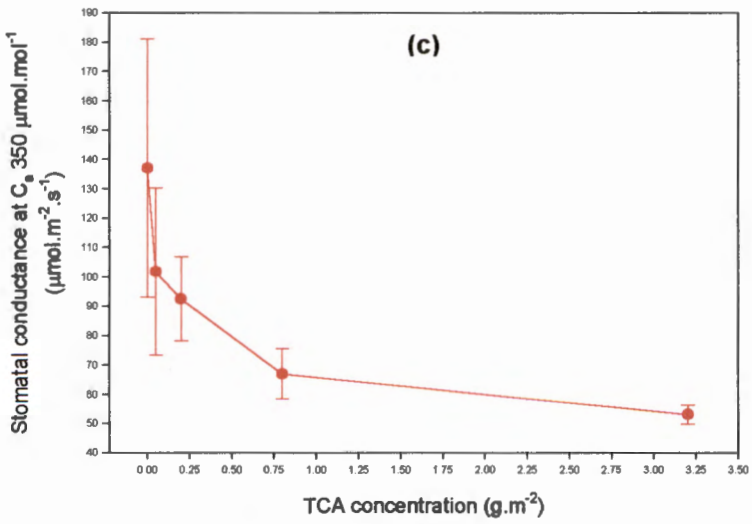


Figure 3.3 Resulting changes in the CO_2 assimilation rate (A) at ambient C_a (a), intercellular CO_2 concentration (C_i) at ambient C_a (b) and stomatal conductance (G_s) at ambient C_a (c) of *Phaseolus vulgaris* after treatment with TCA concentrations of 0.05, 0.20, 0.80 and 3.20 g/m^2. Each data point represents the mean of 4 to 5 replicates.

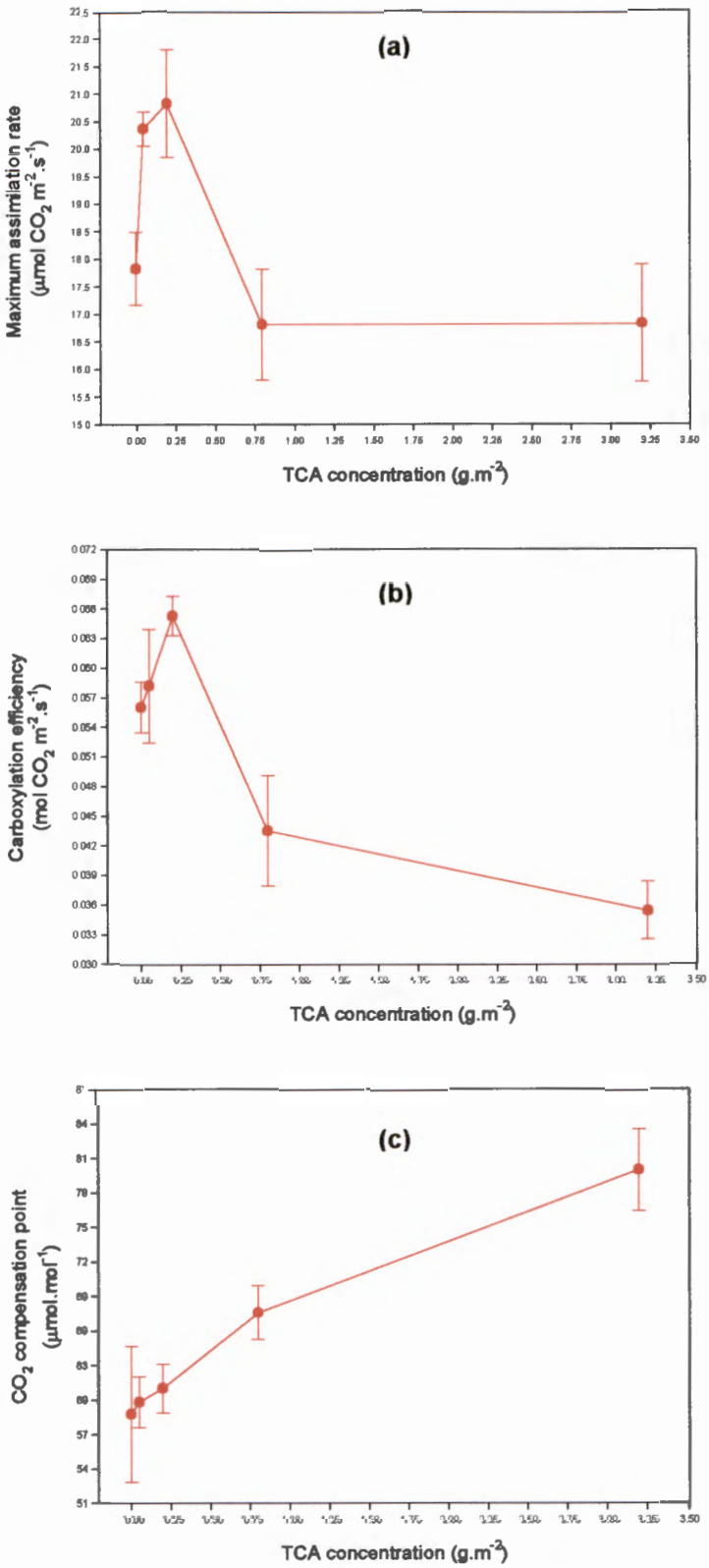


Figure 3.4 Resulting changes in the maximum assimilation rate (A_{max}) (a), carboxylation efficiency (CE) (b) and CO_2 compensation concentration (Γ) (c) of *Phaseolus vulgaris* after treatment with TCA concentrations of 0.05, 0.20, 0.80 and 3.20 g/m^2. Each data point represents the mean of 4 to 5 replicates.

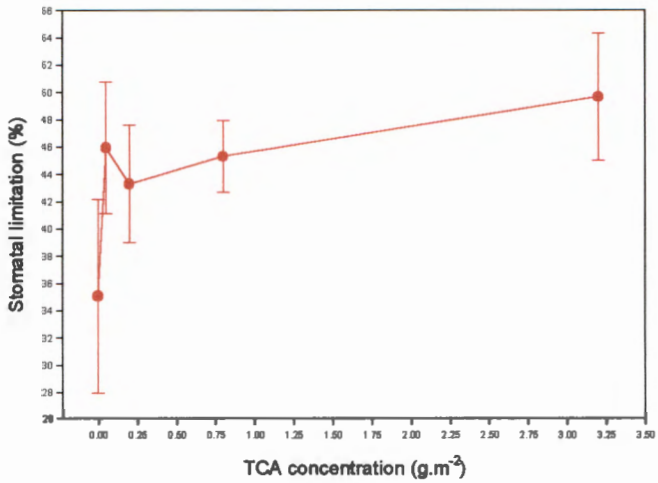


Figure 3.5 Resulting changes in the stomatal limitation (ℓ) of *Phaseolus vulgaris* after treatment with TCA concentrations of 0.05, 0.20, 0.80 and 3.20 g/m^2. Each data point represents the mean of 4 to 5 replicates.

indications that TCA affect photosynthesis by inhibiting mesophyll processes (primary photochemistry and/or enzymatic reactions).

3.1.3.1.2. *Zea mays*:

Figure 3.6 represents the changes in assimilation rate (A) of *Zea mays* as a function of the changes in intercellular CO_2 levels (C_i) as affected by exposure to an increasing range of TCA-concentrations. It is clear that even very low levels of TCA had an effect, since the slope of the response curves decreased with each increase in the TCA-dosage. The changes in the relevant gas exchange parameters brought about by treatment with TCA, are shown in Table 3.2., with indication of the statistical significance.

At the ambient CO_2 concentration of 350 $\mu mol.mol^{-1}$ (C_a), the intercellular CO_2 concentration (C_i) in *Zea mays* showed no significant change upon treatment with a range of different TCA concentrations (Figure 3.7b). The assimilation rate (A_{350}), however, decreased by 19% ($p<0.05$) at 0.05 g TCA/m^2, 23% ($p<0.05$) at 0.20 g TCA/m^2, 49% ($p<0.01$) at 0.80 g TCA/m^2, and 85% ($p<0.01$) at 3.20 g TCA/m^2 (Figure 3.7a). Stomatal conductance (G_s) at ambient C_a showed an initial, but statistically insignificant, increase of 6% at a low TCA concentration of 0.05 g/m^2, after which it decreased, again insignificantly, by 14% at the higher concentration of 0.20 g TCA/m^2. The decreases of 55% and 82% at higher TCA levels of 0.80 and 3.20 g/m^2 respectively, however, were significant ($p<0.01$ in both cases) (Figure 3.7c). TCA exposure caused a decrease in CO_2 assimilation at saturation C_a (A_{max}) (Figure 3.8a). The decreases were as follows: 20% ($p<0.05$) at 0.05 g TCA/m^2, 16% at 0.20 g TCA/m^2, 39% at 0.80 g TCA/m^2 ($p<0.01$), and 72% at 3.20 g TCA/m^2 ($p<0.01$). The initial slope of the demand function ($\delta A/\delta C_i$), representing the carboxylation efficiency (CE), started to decrease significantly at a TCA concentration of 0.20 g/m^2, namely with 35% ($p<0.05$), and subsequently decreased with 46% ($p<0.01$) and 83% ($p<0.01$) at 0.80 and 3.20 g TCA/m^2 respectively (Figure 3.8b). Very low concentrations of TCA had little effect on the stomatal limitation of photosynthesis, while increases of 85% ($p<0.05$), 85% ($p<0.01$) and 95% ($p<0.01$) in stomatal limitation occurred at 0.20, 0.80 and 3.20 g TCA/m^2 respectively (Figure 3.9). No significant changes could be observed in the CO_2 compensation concentration (Γ) after treatment with TCA (Figure 3.8c and Table 3.6).

Although with increasing TCA concentration a decrease in stomatal conductance (G_s) (Figure 3.7c) and resulting increase in stomatal limitation (ℓ) (Figure 3.9), occurred, the value of ℓ remained below 27%, even at the highest TCA concentration used. As was the case with *Phaseolus vulgaris*, the data regarding the decrease in CE (Figure 3.8b) and increase in Γ (Figure 3.8c) overwhelmingly point at mesophyll limitation as the main cause of the decrease in A_{350} (Figure 3.7a) in *Zea mays*, with increasing TCA concentration.

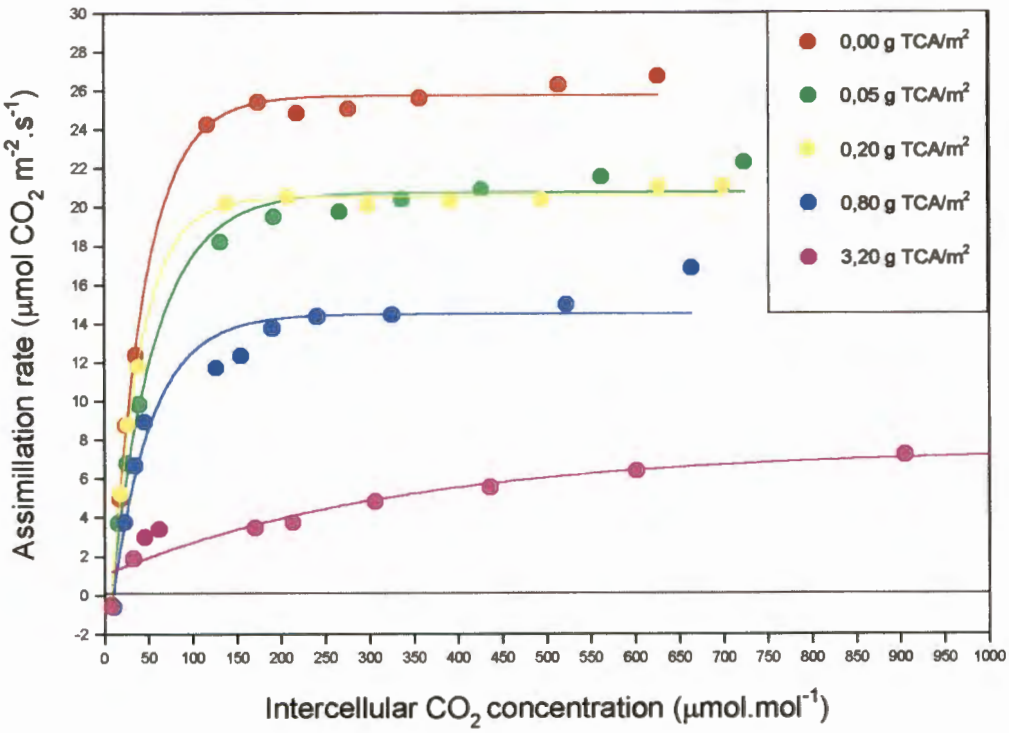


Figure 3.6 CO_2 assimilation rate (A) vs intercellular CO_2 concentration (C_i) response curves for *Zea mays* exposed to TCA concentrations of 0.05, 0.20, 0.80 and 3.20 g/m^2. The plotted values represent the mean of five replicates.

Table 3.2 Rate of CO_2 assimilation (A_{350}) (µmol.$m^{-2}.s^{-1}$) at C_a = 350 µmol.mol^{-1}; intercellular CO_2 concentration (Ci_{350}) (µmol.mol^{-1}) at C_a = 350 µmol.mol^{-1}; stomatal conductance (Gs_{350}) (m.s^{-1}) at C_a = 350 µmol.mol^{-1}; maximum rate of CO_2 assimilation (A_{max}) (µmol.$m^{-2}.s^{-1}$); initial slope of the demand function ($\delta A/\delta C_i$) (mol.$m^{-2}.s^{-1}$); CO_2 compensation point (Γ) (µmol.mol^{-1}) and stomatal limitation of photosynthesis (ℓ) (%) in *Zea mays* treated with a range of TCA concentrations, with * and ** indicating significant differences ($p<0.05$ and $p<0.01$ respectively) compared to untreated plants.

	Untreated	0.05 g TCA/m^2	0.20 g TCA/m^2	0.80 g TCA/m^2	3.20 g TCA/m^2
A_{350}	25.4±1.81	20.53±0.303*	19.5±0.77*	12.98±0.69**	3.725±0.52**
Ci_{350}	174.5±11.72	208±17.91	192.25±8.34	159.25±12.05	213±17.34
Gs_{350}	265.25±24.82	283.25±36.34	229.25±22.26	119.25±11.61**	48.75±6.02**
A_{max}	26.7±1.74	21.35±0.087*	22.525±0.42	16.33±0.32**	7.53±0.66**
$\delta A/\delta C_i$	0.497±0.058	0.409±0.036	0.324±0.025*	0.268±0.013**	0.083±0.029**
Γ	9.25±2.2	8.5±1.5	9.67±0.88	11.88±0.52	13.25±3.54
ℓ	1.32±0.33	0.74±0.42	8.567±1.66*	8.86±1.96*	26.37±4.69*

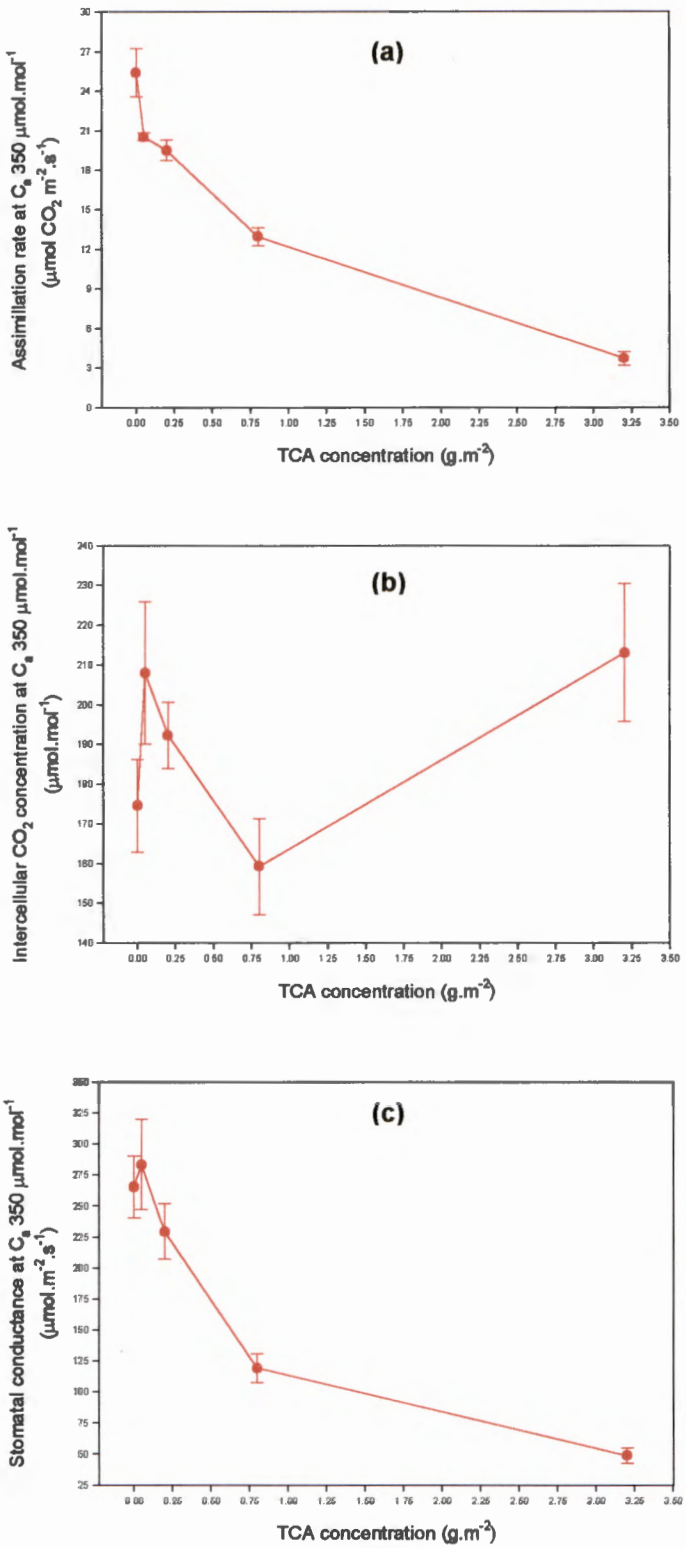


Figure 3.7 Resulting changes in the CO_2 assimilation rate (A) at ambient C_a (a), intercellular CO_2 concentration (C_i) at ambient C_a (b) and stomatal conductance (G_s) at ambient C_a (c) of *Zea mays* after treatment with TCA concentrations of 0.05, 0.20, 0.80 and 3.20 g/m^2. Each data point represents the mean of 4 to 5 replicates.

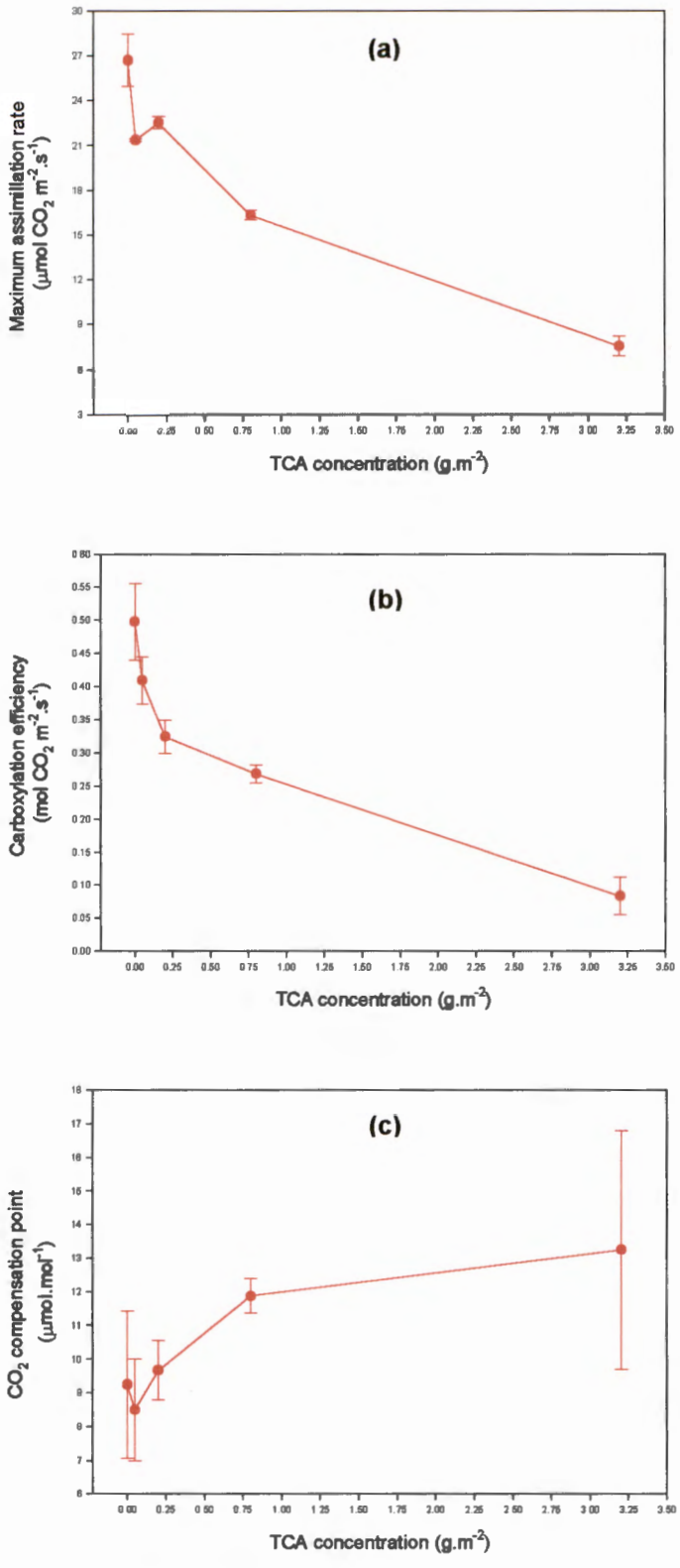


Figure 3.8 Changes in the maximum assimilation rate (A_{max}) (a), carboxylation efficiency (CE) (b) and CO_2 compensation concentration (Γ) (c) of *Zea mays* after treatment with TCA concentrations of 0.05, 0.20, 0.80 and 3.20 g/m^2 respectively. Each data point represents the mean of 4 to 5 replicates.

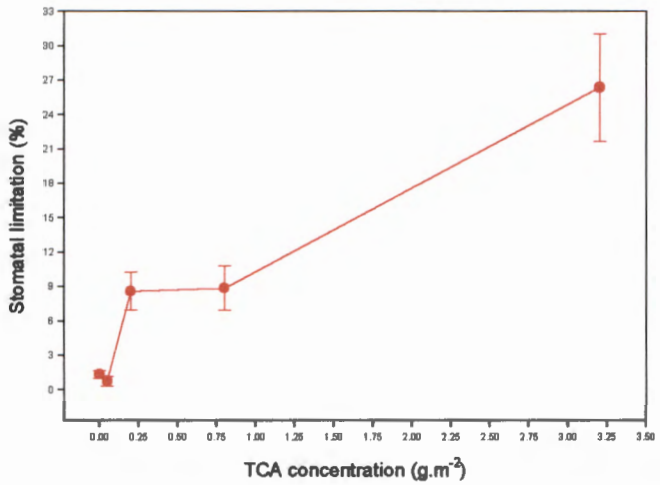


Figure 3.9 Changes in the stomatal limitation (ℓ) of *Zea mays* after treatment with TCA concentrations of 0.05, 0.20, 0.80 and 3.20 g/m^2. Each data point represents the mean of 4 to 5 replicates.

No initial increase at low TCA concentration, as was the case with *Phaseolus vulgaris*, could be observed in the activity of any of the CO_2 gas exchange parameters upon treatment with TCA. Thus even the lowest TCA concentration used had an inhibitory effect on the metabolism of *Zea mays*. TCA was formerly used as a herbicide to control monocotyledonous weeds (Brian, 1976), and would be expected to act on *Zea mays* even at very low concentrations.

3.1.3.2. Effect of TECE on photosynthetic CO_2-gas exchange:

3.1.3.2.1. *Phaseolus vulgaris*:

A marked TECE-induced decrease occurred in the initial slope of the A:C_i response curve (CO_2 assimilation rate (A) vs. intercellular CO_2 concentration (C_i)) (Figure 3.10) of *Phaseolus vulgaris*. The changes in photosynthetic gas exchange parameters are presented in Table 3.3.

Exposure to TECE had a significant effect on certain parameters of CO_2 assimilation. After one week of exposure to 810 µg/m^3 TECE, the following changes occurred at an ambient CO_2 concentration (C_a) of 350 µmol.mol^{-1}: (i) the rate of CO_2 assimilation (A) decreased by 83% ($p<0.01$) (Figure 3.11a); (ii) a 78% ($p<0.05$) decrease occurred in stomatal conductance (Figure 3.11c); (iii) a 6% increase in the intercellular CO_2 concentration (C_i) could be observed, although the change was not statistically significant (Figure 3.11b); (iv) there was a 42% ($p<0.01$) decrease in the maximum rate of CO_2 assimilation (Figure 3.12a); (v) the initial slope of the demand function ($\delta A/\delta C_i$), representing the carboxylation efficiency, decreased by 77% ($p<0.01$) (Figure 3.12b); (vi) the stomatal limitation of photosynthesis (ℓ) increased with 44% ($p<0.05$) (Figure 3.13), and (vii) no significant change could be observed in the CO_2 compensation concentration (Γ) (Figure 3.12c).

Although an increase occurred in stomatal limitation (from 34% to 61%), the data clearly indicate that mesophyll processes were inhibited by the TECE fumes, since the CE ($\delta A/\delta C_i$) decreased largely. The increase in C_i at 350ppm is also an indication of reduced Rubisco activity.

Exposure to TECE thus had a definite inhibitory effect on the gas exchange of *Phaseolus vulgaris*. This is corroborated by the decrease, although very small, observed in the vitality index (PI_{ABS}) (3.2.2.2.1.), as well as the decrease in the activity of Rubisco (3.3.2.2.1.).

3.1.3.2.2. *Zea mays*:

After one week treatment at 810 µg TECE/m^3, no change was evident in the initial slope of the A:C_i response curves relative to the control, although the level at which the CO_2 assimilation rate of the treated plants became saturated was lower than that of the control (Figure 3.14).

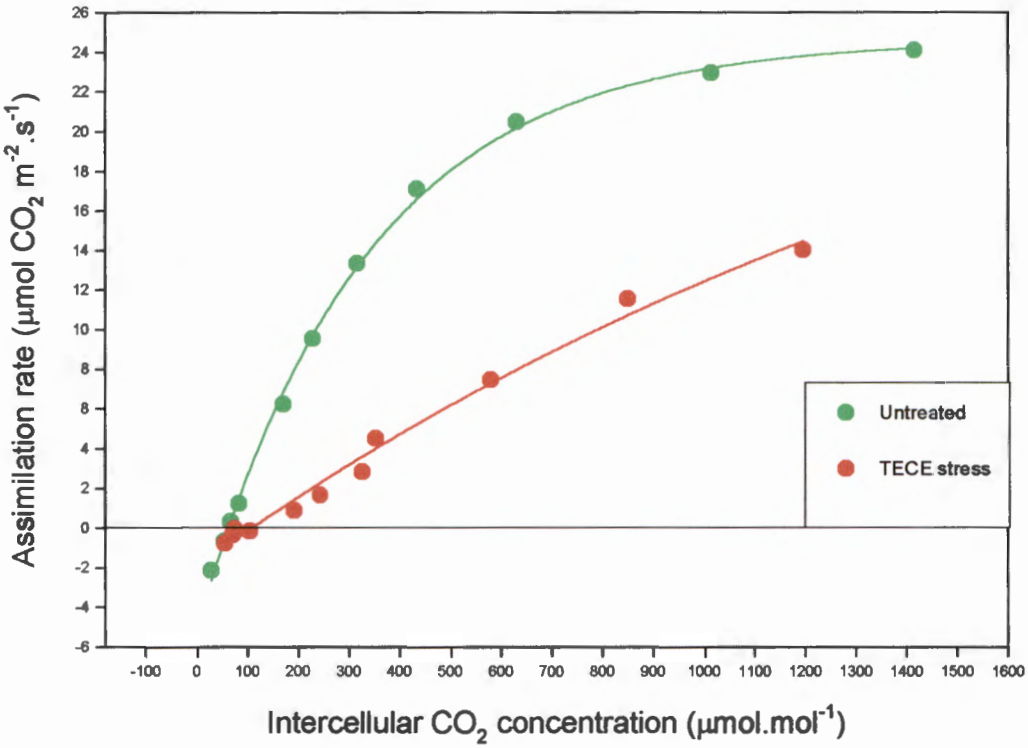


Figure 3.10 CO_2 assimilation rate (A) vs intercellular CO_2 concentration (C_i) response curves for *Phaseolus vulgaris* exposed to a TECE concentration of 810 µg/m^3 for a period of one week, with the plotted values representing the mean of four replicates.

Table 3.3 Rate of CO_2 assimilation (A_{350}) (µmol.m^{-2}.s^{-1}) at C_a = 350 µmol.mol^{-1}; intercellular CO_2 concentration (Ci_{350}) (µmol.mol^{-1}) at C_a = 350 µmol.mol^{-1}; stomatal conductance (Gs_{350}) (m.s^{-1}) at C_a = 350 µmol.mol^{-1}; maximum rate of CO_2 assimilation (A_{max}) (µmol.m^{-2}.s^{-1}); initial slope of the demand function ($\delta A/\delta C_i$) (mol.m^{-2}.s^{-1}); CO_2 compensation point (Γ) (µmol.mol^{-1}) and stomatal limitation of photosynthesis (ℓ) (%) for *Phaseolus vulgaris* treated with a TECE for a period of one week, with * and ** indicating significant differences ($p<0.05$ and $p<0.01$ respectively) compared to untreated plants.

	Untreated	TECE stress
A_{350}	9.54±1.64	1.66±0.45**
Ci_{350}	227.8±10.59	242.6±12.46
Gs_{350}	125.25±24.49	28±3.77*
A_{max}	24.1±0.902	14.02±0.664**
$\delta A/\delta C_i$	0.057±0.007	0.013±0.0035**
Γ	67±3.39	86.25±11.43
ℓ	34.43±7.05	61.57±4.86*

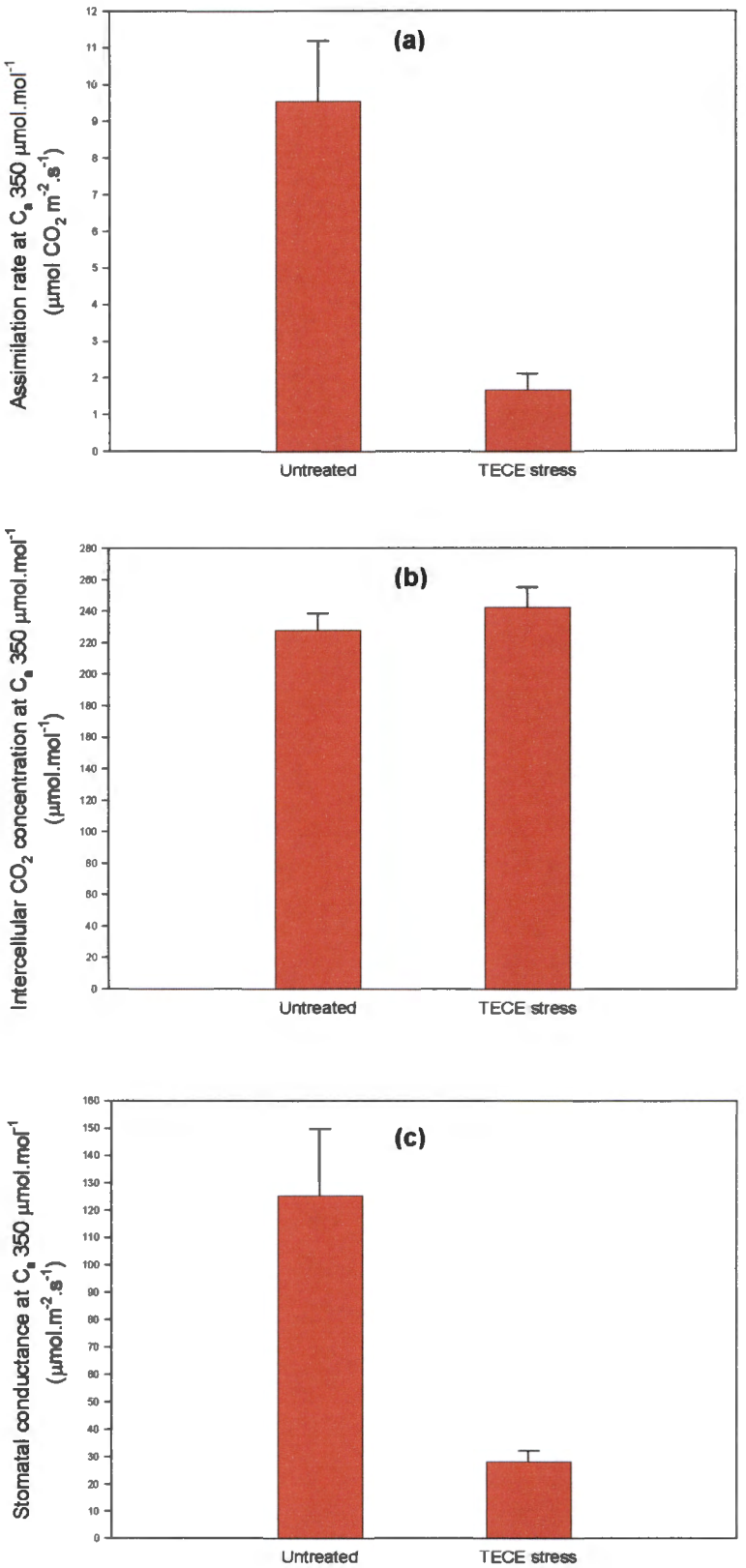


Figure 3.11 Change in the CO_2 assimilation rate (A) at ambient C_a (a), intercellular CO_2 concentration (C_i) at ambient C_a (b) and stomatal conductance (G_s) at ambient C_a (c) of *Phaseolus vulgaris* as a result of seven days exposure to 810 µg TECE/m^3. Each data point represents the mean of four replicates.

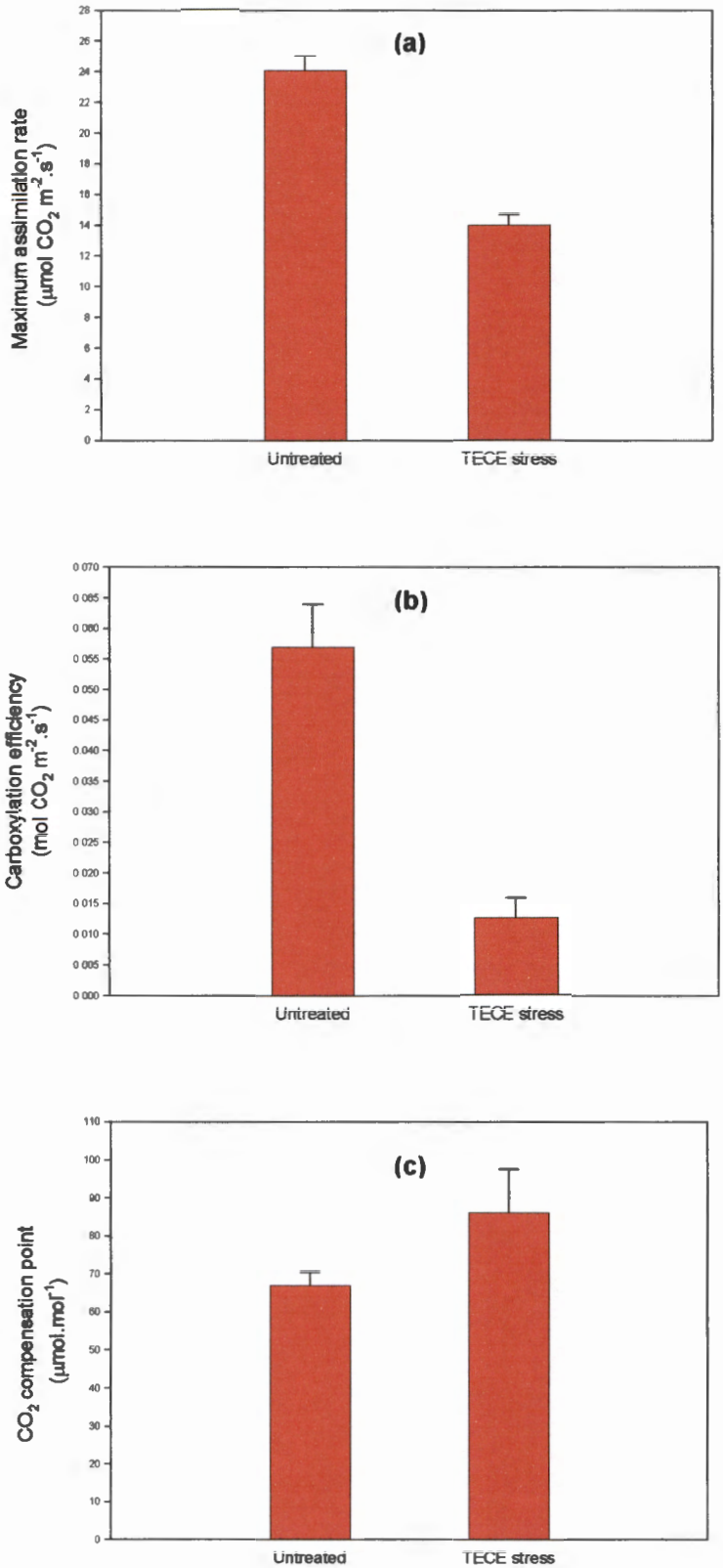


Figure 3.12 Change in the maximum assimilation rate (A_{max}) (a), carboxylation efficiency (CE) (b) and CO_2 compensation concentration (Γ) (c) of *Phaseolus vulgaris* as a result of seven days exposure to 810 µg TECE/m^3. Each data point represents the mean of four replicates.

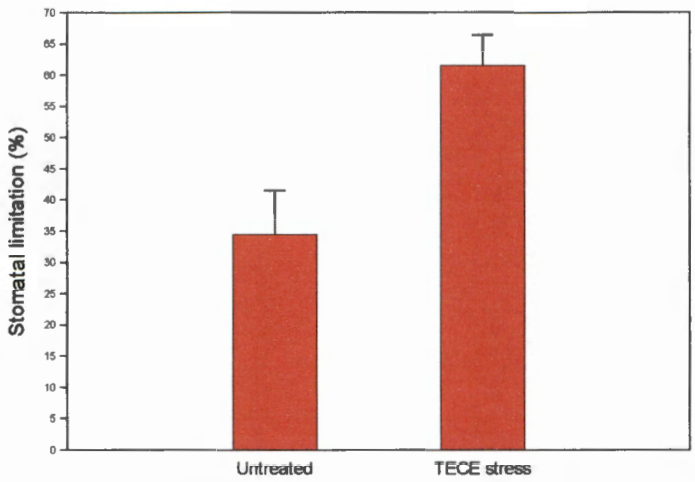


Figure 3.13 Change in the stomatal limitation (ℓ) of *Phaseolus vulgaris* as a result of seven days exposure to 810 µg TECE/m^3. Each data point represents the mean of four replicates.

Although some of the photosynthetic gas exchange parameters of *Zea mays* showed changes after a week of exposure to TECE, none of these changes were statistically significant, as can be seen from the data shown in Table 3.4. and Figures 3.15 to 3.17.

According to photosynthetic gas exchange data, it seems that *Zea mays*, which is a C_4-plant, was much more resistant to the TECE fumes than the C_3-plant *Phaseolus vulgaris*, at least for the duration of the experiment. This probably must be ascribed to the special leaf anatomy of the C_4 leaf and the fact that PEP carboxylase is the initial enzyme that fixes CO_2 (Edwards and Walker, 1983). Rubisco, which according to the data obtained with *P. vulgaris* is strongly inhibited by TECE, is shielded from the outside atmosphere in *Zea mays* due to its location in the tightly packed bundle sheath cells.

3.2. Chlorophyll Fluorescence:

3.2.1. Quantification of fluorescence transients: The JIP-test:

3.2.1.1. Fluorescence emission:

Part of the light energy absorbed by the pigments (mostly chlorophyll) is lost by re-emission as fluorescence or as heat. Since the decay processes of excited chlorophyll are competitive, changes in photosynthetic rate and/or dissipative heat will lead to complementary changes in emitted fluorescence intensity (Bolhàr-Nordenkampf and Öquist, 1993).

One photon of red light contains enough energy for a chlorophyll molecule to reach the first excited state, but it remains stable for less than 10^{-8} seconds, during which charge separation takes place within the reaction centre, forming the primary photochemical step of photosynthesis. If no charge separation occurs, the absorbed energy is released as fluorescence and/or heat when the excited molecule returns to the original energy level.

The three decay processes photochemistry (P), fluorescence (F) and heat dissipation resulting in de-excitation of excited chlorophyll molecules (D), have a relationship which is best expressed using rate constants, namely k_P, k_F and k_D. Thus in one second the total number of de-excitations is:

$$(k_P + k_F + k_D)n,$$

where n is the number of chlorophyll molecules. At room temperature all fluorescence originates from chlorophyll molecules associated with PS II, thus the yield (Φ) of PS II fluorescence can be defined as

$$\Phi F = k_F/(k_P + k_F + k_D)$$

under constant illumination conditions (Bolhàr-Nordenkampf and Öquist, 1993).

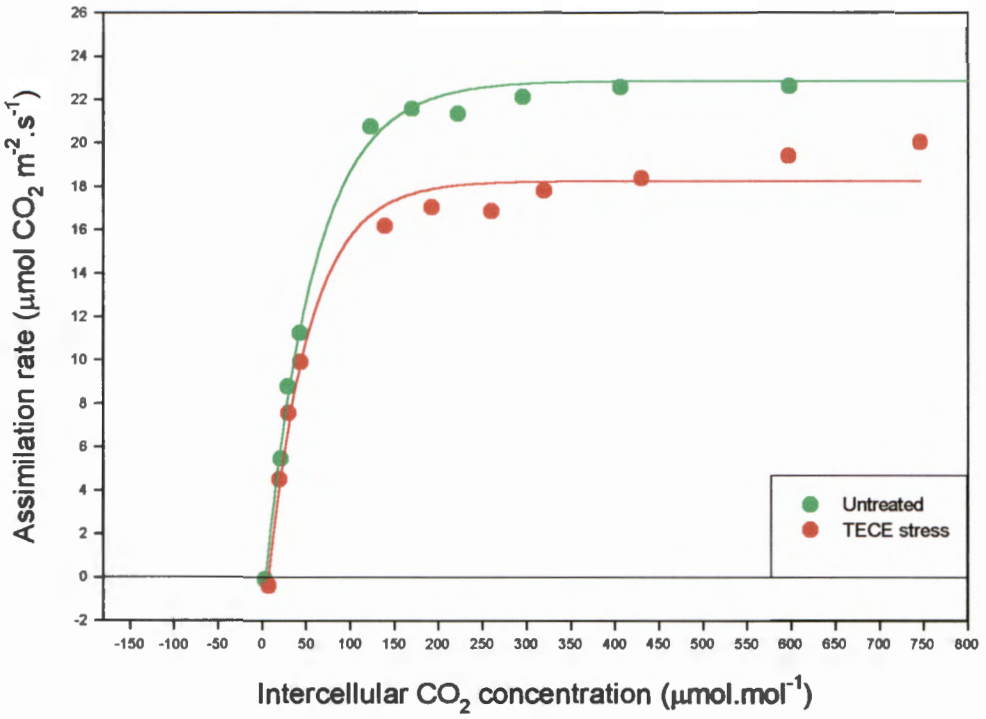


Figure 3.14 CO_2 assimilation rate (A) vs intercellular CO_2 concentration (C_i) response curves for *Zea mays* exposed to a TECE concentration of 810 µg/m^3 for a period of one week. Plotted values represent the mean of four replicates.

Table 3.4 Rate of CO_2 assimilation (A_{350}) (µmol.m^{-2}.s^{-1}) at C_a = 350 µmol.mol^{-1}; intercellular CO_2 concentration (Ci_{350}) (µmol.mol^{-1}) at C_a = 350 µmol.mol^{-1}; stomatal conductance (Gs_{350}) (m.s^{-1}) at C_a = 350 µmol.mol^{-1}; maximum rate of CO_2 assimilation (A_{max}) (µmol.m^{-2}.s^{-1}); initial slope of the demand function ($\delta A/\delta C_i$) (mol.m^{-2}.s^{-1}); CO_2 compensation point (Γ) (µmol.mol^{-1}) and stomatal limitation of photosynthesis (ℓ) (%) for *Zea mays* treated with a TECE for a period of one week.

	Untreated	**TECE stress**
A_{350}	21.55±0.94	17.025±2.42
Ci_{350}	191.67±13.45	215.33±17.68
Gs_{350}	230.25±39.16	231.5±67.23
A_{max}	24.53±0.86	20.05±1.88
$\delta A/\delta C_i$	0.286±0.017	0.280±0.017
Γ	5.0±2.2	2.67±1.45
ℓ	7.77±1.64	2.91±1.65

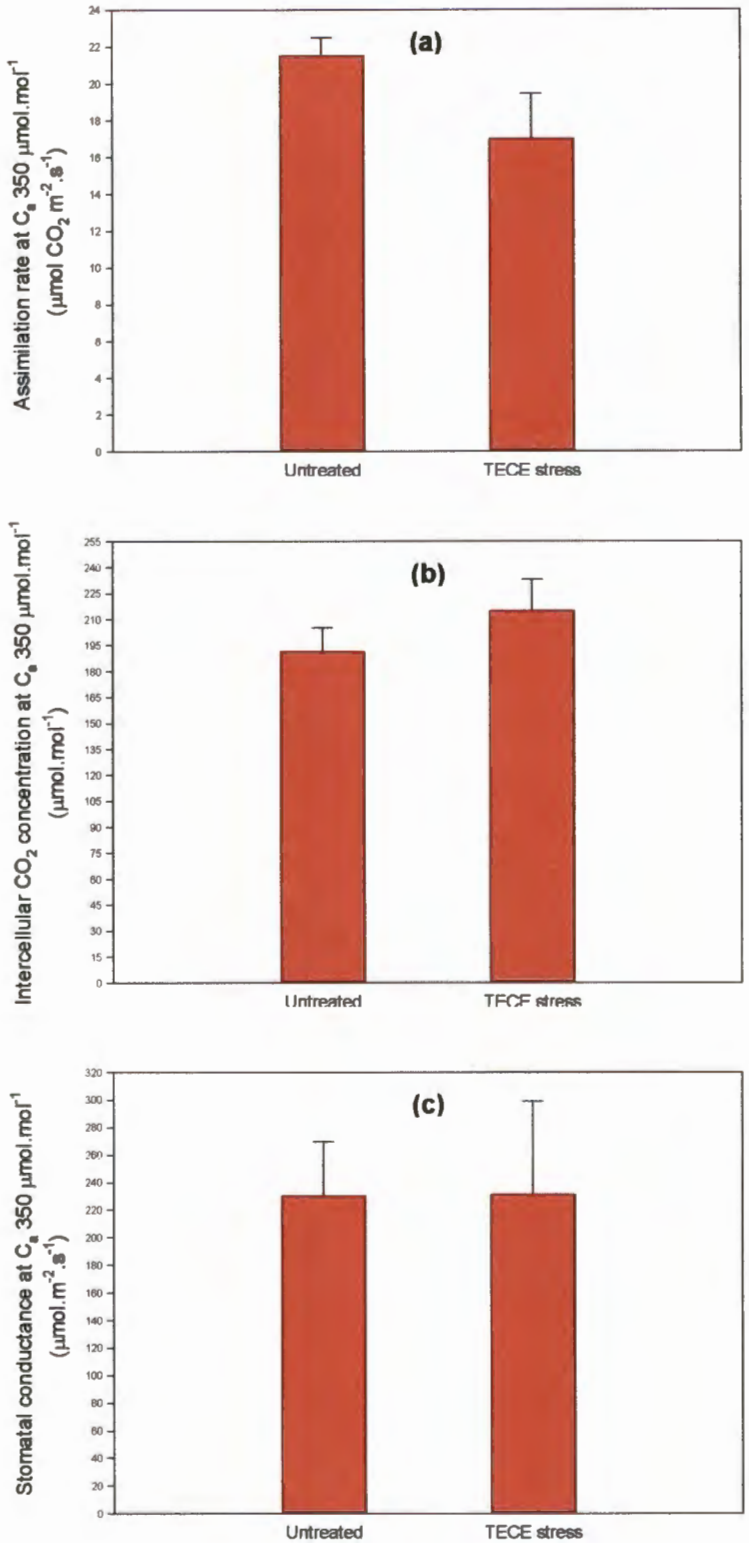


Figure 3.15 Change in the CO_2 assimilation rate (A) at ambient C_a (a), intercellular CO_2 concentration (C_i) at ambient C_a (b) and stomatal conductance (G_s) at ambient C_a (c) in *Zea mays* as a result of seven days exposure to 810 µg TECE/m^3. Each data point represents the mean of four replicates.

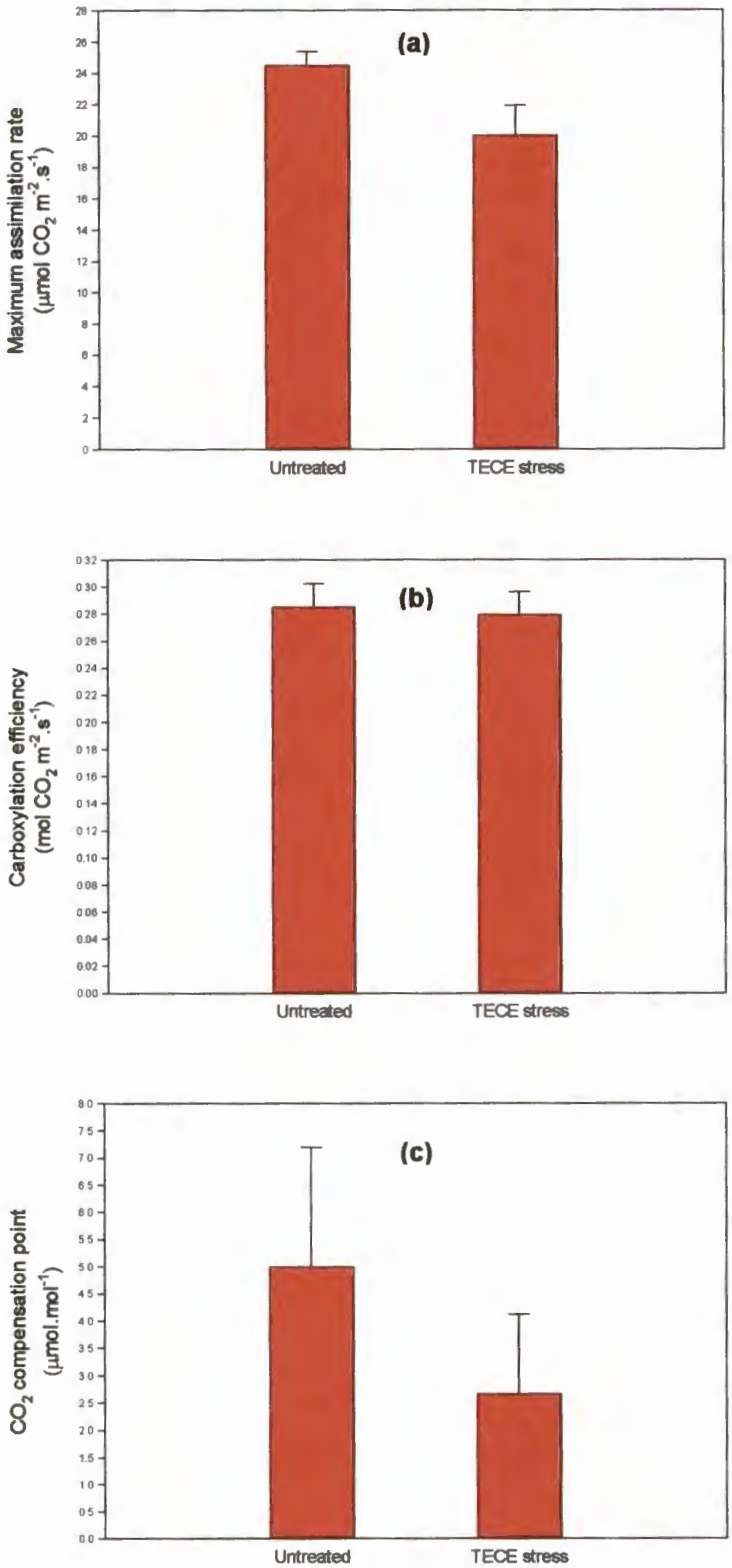


Figure 3.16 Change in the maximum assimilation rate (A_{max}) (a), carboxylation efficiency (CE) (b) and CO_2 compensation concentration (Γ) (c) of *Zea mays* as a result of seven days exposure to 810 µg TECE/m^3. Each data point represents the mean of four replicates.

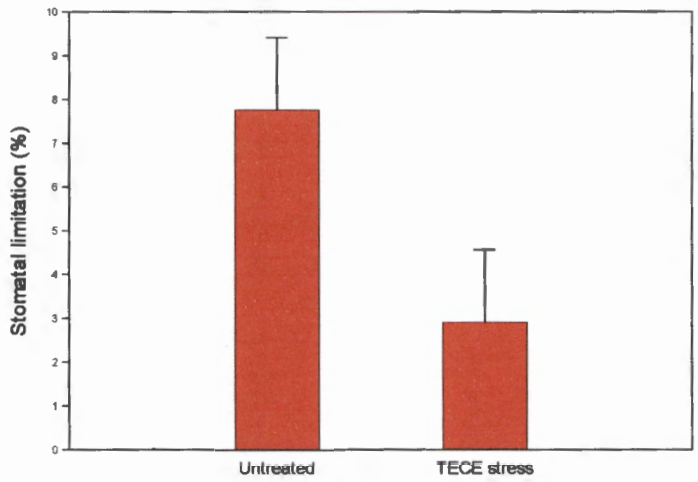


Figure 3.17 Change in the stomatal limitation (ℓ) of *Zea mays* as a result of seven days exposure to 810 µg TECE/m^3. Each data point represents the mean of four replicates.

3.2.1.2. Fluorescence transients:

The fluorescence transient, also called the Kautsky transient (Kautsky *et al.*, 1960), shows a fast rise completed in less than one second, and subsequent slower decline towards a steady state (Strasser *et al.*, 2001), and consists of different phases denoted as O K J I D P S M T. The rise from O to P is called the 'fast phase' and takes place within the first second of illumination, is rich in information and is related to the primary photochemical processes of PSII. The 'slow phase' follows after P, reaching the terminal phase, T, after several minutes. The 'slow phase' is mainly related to interactions between the processes in the thylakoid membranes and metabolic processes in the stroma (Bolhàr-Nordenkampf and Öquist, 1993).

The fast rise reflects the accumulation of the reduced form of the primary quinone acceptor Q_A, i.e. closure of reaction centres (RC), which is the net result of Q_A reduction due to PSII activity and Q_A^- reoxidation due to photosystem I (PSI) activity. A few minutes exposure to darkness usually fully oxidises Q_A, leaving all the RC's open. The fluorescence yield at the onset of illumination is denoted as F_0. The maximum yield F_P at the end of the rise reaches its maximum possible value (F_M) if illumination is strong enough to ensure that all RC's are closed (Schreiber *et al.*, 1995; Strasser *et al.*, 2001). In some cases the full transient shows a sequence of more steps, e.g. F_0-J-I-H-G (Tsimilli-Michael *et al.*, 1998) with an additional step K appearing at 300 µs in heat-stressed samples, forming a transient F_0-K-J-I-P (Srivastava *et al.*,1997).

The physiological state of a sample at the moment of measuring determines the shape of the fluorescence transient. The prevailing physical and chemical environmental conditions around the sample are also contributing factors. Furthermore the actual physiological state of a sample is a function of the states the sample went through prior to the measurements (Krüger *et al.*, 1997).

Thus the fluorescence transient can be applied by using well-defined plant material under well-defined physical conditions to analyse its response to chemical changes in the environment (Strasser *et al.*, 2000). In this investigation the effect of TCA and TECE treatments on *Phaseolus vulgaris* and *Zea mays* was quantified by the JIP-test (Strasser and Strasser, 1995).

3.2.1.3. The JIP-test:

The polyphasic fluorescence rise during the first second of illumination includes phases labelled O, J, I and P (Figure 3.18), hence the name 'JIP-test'. The transient changes its shape in accordance to environmental conditions, and provides information about the structure, conformation and function of the photosynthetic apparatus (Strasser *et al.*, 1996).

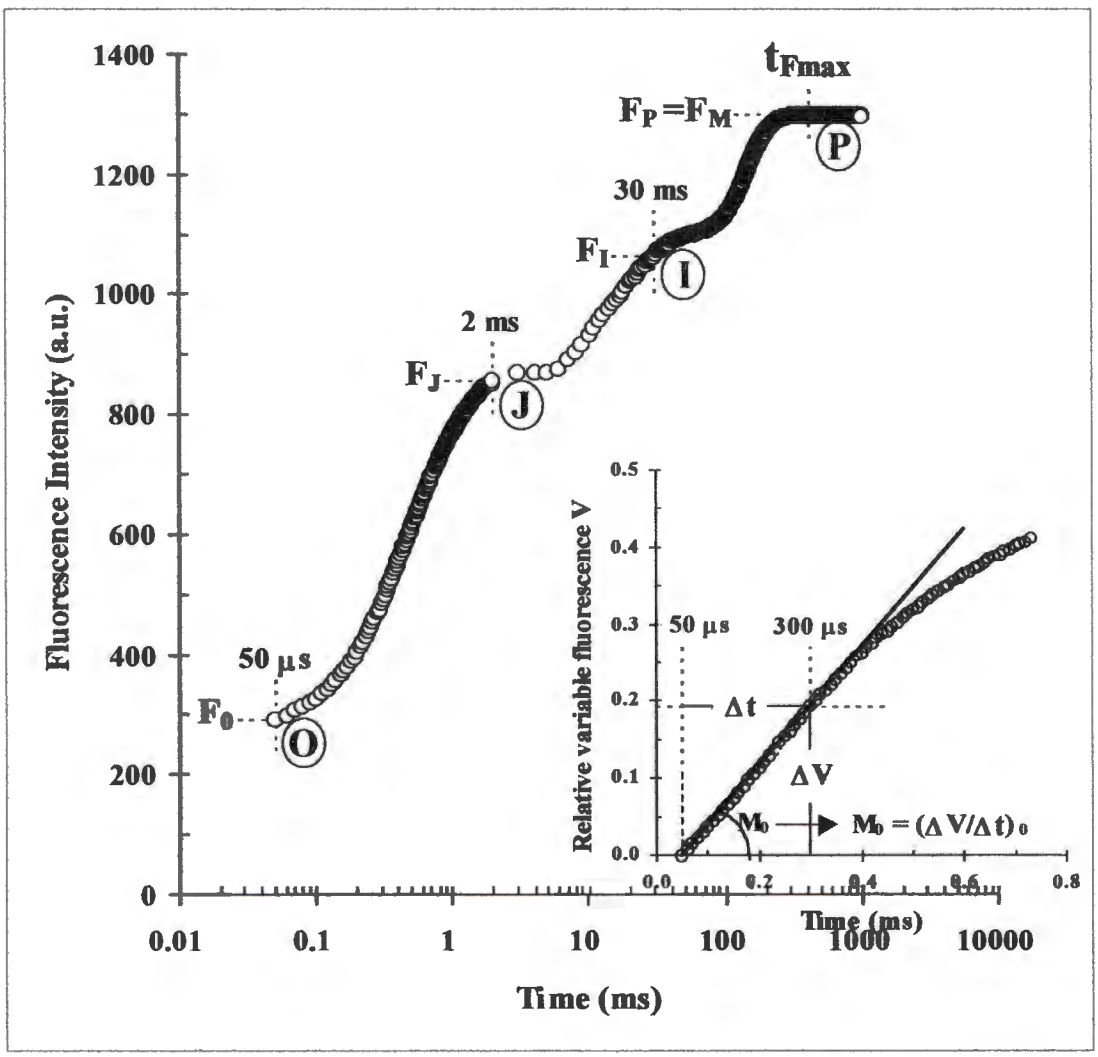


Figure 3.18 The transient shows a Chl *a* polyphasic fluorescence rise O-J-I-P, plotted on a logarithmic time scale from 50 µs to 1 s (Strasser and Tsimilli-Michael, 2001).

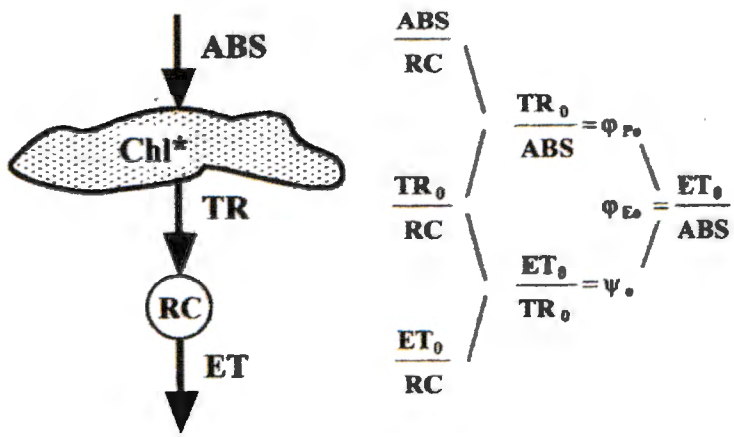


Figure 3.19 Simplified scheme demonstrating the energy cascade from PSII light absorption to electron transport (Strasser and Strasser, 1995).

The following values were selected from the original data stored during the first second of illumination, and used for calculation of several phenomenological and biophysical expressions leading to the dynamic description of a photosynthetic sample at a given physiological state:

- the maximal measured fluorescence intensity F_P, provided that the excitation energy is high enough to permit the closure of all RC's so that $F_P = F_M$;
- the fluorescence intensity at 50 µs (minimal fluorescence), considered to be the intensity when all RC's are open (F_0);
- the fluorescence intensity at 150 µs and at 300 µs;
- the fluorescence intensity at 2 ms and 30 ms, denoted as F_J and F_I respectively;
- the time (t_{Fmax}) to reach the maximal fluorescence intensity;
- the area between the fluorescence transient and the level of the maximal fluorescence intensity, $F = F_M$.

Any step in the transient could however be the highest and then subsequently become F_P or even F_M if all RC's are closed (Krüger *et al.*, 1997).

The O-J part of a transient is considered as the photochemical phase, i.e. the phase reflecting single turn over events (Q_A reduction by PSII activity), while the J-P phase reflects multiple turn over events, i.e. Q_A^- accumulation as the net result of Q_A reduction by PSII and Q_A^- reoxidation by the electron transport chain (Strasser *et al.* 1995, Van Heerden *et al.*, 2003).

This distinction is supported by the finding that the *in vivo* exhibited O-J phase coincides with the monophasic (O-J-I-P) fluorescence rise exhibited in the presence of an inhibitor of Q_A^- reoxidation (e.g. DCMU: 3-(-3,4-dichlorophenyl)-1,1-dimethylurea), when both are normalised from 0 to 1 (Strasser and Strasser, 1995).

Optimisation of the time at point J, i.e. the lowest possible slope or if existing, the time at slope zero, determined a fixed time of 2 ms to be used for the J step. If 1 or 3 ms is used the value would show a very small change. The JIP-test uses and depends on the digital calculation of the slope at the origin of the fluorescence transient from time zero to time j (e.g. 2 ms). The maximal rate of primary photochemistry is proportional to this slope, dF/dt_0, or if normalised, dV/dt_0. If this variable transient is normalised to the J-step, it becomes Vt/V_J, which represents the single turn over domain for time 0 to time J (e.g. 2 ms) with or without DCMU. Consequently the maximal fractional velocity of Q_A reduction is represented by the normalised original slope $dV/dt_0/V_J$, which equals $dQ_A/Q_{A,total}/dt_0$ (Strasser *et al.*,1995).

Figure 3.19 shows a simplified working model of the energy fluxes in the photosynthetic apparatus. Experimental values obtained with the JIP-test were used to derive the formulae, shown in Table 3.5, for the calculation of the specific energy fluxes (per RC) and

Table 3.5 A summary of the JIP-test formulae using data extracted from the fast fluorescence transient O-J-I-P (Strasser and Tsimilli-Michael, 2001).

Extracted and technical fluorescence parameters		
F_0	=	$F_{50\mu s}$, fluorescence intensity at 50μs
F_{150}	=	fluorescence intensity at 150μs
F_{300}	=	fluorescence intensity at 300μs
F_J	=	fluorescence intensity at the J-step (at 2ms)
F_M	=	maximal fluorescence intensity
t_{Fmax}	=	time to reach F_M, in ms
V_J	=	$(F_{2ms} - F_0) / (F_M - F_0)$
Area	=	area between fluorescence curve and F_M
$(dV/dt)_0$ or M_0	=	$4 . (F_{300} - F_0) / (F_M - F_0)$
S_m	=	Area / $(F_M - F_0)$
B_{av}	=	$1 - (S_m / t_{Fmax})$
N	=	$S_m . M_0 . (1 / V_J)$ turn over number of Q_A
Quantum efficiencies or flux ratios		
φ_{Po} or TR_0 / ABS	=	$1 - (F_0 / F_M)$ or F_V / F_M
φ_{Eo} or ET_0 / ABS	=	$[1 - (F_0 / F_M)] . \psi_0$
ψ_o or ET_0 / TR_0	=	$1 - V_J$
Specific fluxes or specific activities		
ABS/RC	=	$M_0 . (1/V_J) . (1/\varphi_{Po})$
TR_0/RC	=	$M_0 . (1/V_J)$
ET_0/RC	=	$M_0 . (1/V_J) . \psi_0$
DI_0/RC	=	$(ABS/RC) - (TR_0/RC)$
Phenomenological fluxes or phenomenological activities		
ABS/CS_0	=	F_0 or an other useful expression *
TR_0/CS_0	=	$\varphi_{Po} . (ABS / CS_0)$
ET_0/CS_0	=	$\varphi_{Po} . \psi_0 . (ABS/CS_0)$
DI_0/CS_0	=	$(ABS/CS_0) - (TR_0/CS_0)$
Density of reaction centres		
RC/CS_0	=	$\varphi_{Po} . (V_J/M_0) . F_0$ *
Performance index		
PI_{ABS}	=	$(RC/ABS) . [\varphi_{Po}/(1-\varphi_{Po})] . [\psi_0/(1-\psi_0)]$
Driving forces		
DF_{ABS}	=	log $[PI_{ABS}]$
DF_{RC}	=	log [RC/ABS]
$DF\varphi$	=	log $[\varphi_{Po}/(1-\varphi_{Po})]$
$DF\psi$	=	log $[\psi_o/(1-\psi_o)]$
* when expressed per CS_M, F_0 is replaced by F_M		

phenomenological energy fluxes (per excited cross-section CS), as well as for the flux ratios or yields. The derivation was done based on the theory of energy fluxes in biomembranes (Strasser *et al.*, 1995). At any instant the constellation of their values can be considered an expression of the systems behaviour (Krüger *et al.*, 1997).

ABS refers to the flux of photons absorbed by the antenna pigments (Chl^* denotes excited chlorophyll). Part of this excitation energy is dissipated, mainly as heat and less as fluorescence emission, F, while another part is channelled as trapping flux (TR) to the reaction centre RC. There the excitation energy is converted to redox energy by reducing the electron acceptor Q_A to Q_A^-, which is then reoxidised to Q_A thus creating an electron transport, ET, that leads ultimately to CO_2-fixation (Krüger *et al.*, 1997; Strasser *et al.* 2000).

The flux ratios or yields derived include the maximum quantum yield of primary photochemistry ($\varphi_{P0} = TR_0/ABS$), the efficiency ($\Psi_0 = ET_0/TR_0$) with which a trapped exciton can move an electron into the electron transport chain further than Q_A^-, and the quantum yield of electron transport ($\varphi_{E0} = ET_0/ABS = \varphi_{P0} \cdot \Psi_0$) (Strasser *et al.*, 2001). These yields are directly related to the three specific energy fluxes at the onset of excitation (time zero) ABS/RC, TR_0/RC and ET_0/RC, as the ratios of any two of them. TR/RC is the rate by which an exciton is trapped by the RC resulting in reduction of Q_A to Q_A^-. TR_0/RC expresses the maximal value of this rate, since all RC's are open at time zero. Derivation of the TR_0/RC link of the specific trapping flux with experimental data is explained by Strasser *et al.*, 2000. TR_0/RC expresses the initial rate of RC-closure as a fractional expression over the total number of RCs that can be closed. However, under stress conditions, some RCs are inactivated in the sense of being transformed to quenching sinks without Q_A-reduction. Then TR_0/RC still only refers to active RCs. Since the derivation of the other two specific fluxes is also based on TR_0/RC, the same is valid for them. (Strasser *et al.*, 2000).

Accordingly the phenomenological fluxes for the excited cross-section are ABS/CS, TR_0/CS and ET_0/CS. A measure of the phenomenological absorption flux ABS/RC is provided by the value of the initial fluorescence F_0. For further derivation of the phenomenological fluxes refer to Strasser *et al.*, 2000.

Expressions relating the photochemical rate constant k_P and the nonphotochemical rate constant k_N summing up k_H (heat dissipation), k_F (fluorescence emission) and k_X (energy migration to PSII) with the fluorescence values F_0 and F_M have been derived based on the theory of energy fluxes in biomembranes (Krüger *et al.*, 1997).
Thus:

$$k_N = (ABS/CS) \cdot k_F \cdot (1/F_M)$$

$$k_N + k_P = (ABS/CS) \cdot k_F \cdot (1/F_0)$$

$$k_P = (ABS/CS) \cdot k_F \cdot \{(1/F_0) - (1/F_M)\}$$

Leading to:

$$\varphi_{P0} = 1 - (F_0/F_M) = k_P/(k_P + k_N)$$

3.2.1.4. The Performance Index (PI):

The performance index is a multiparametric expression which takes into consideration the absorption of light energy (chlorophyll concentration), the quantum efficiency of primary photochemistry (trapping) and the efficiency (probability) of the conversion of excitation to electron transport in the electron transport chain of the thylakoid membrane.

The performance index PI (Strasser *et al.*, 2000), calculated on absorption basis, (PI_{ABS}), is presented as:

$$PI_{ABS} = \gamma_{RC}/(1 - \gamma_{RC}) \cdot \varphi_{Po}/(1 - \varphi_{Po}) \cdot \Psi_o/(1 - \Psi_o)$$
$$= RC/ABS \cdot \varphi_{Po}/(1 - \varphi_{Po}) \cdot \Psi_o/(1 - \Psi_o)$$

where γ_{RC} is the fraction of reaction centre chlorophylls relatively to the total chlorophyll:

$$\gamma_{RC} = Chl_{RC}/Chl_{total}$$

Since $Chl_{tot} = Chl_{antenna} + Chl_{RC}$, $\gamma_{RC}/(1 - \gamma_{RC}) = Chl_{RC}/Chl_{antenna} = RC/ABS$.

By definition the performance index is a product of expressions of the form $[p_i/(1 - p_i)]$, where p_i stands for fraction probabilities. If p_i represents the fraction of the reduced and $(1 - p_i)$ the fraction of the oxidised form of a compound, then $\log[p_i/(1 - p_i)]$ expresses the potential or driving force for the corresponding oxido-reduction reaction (according to the Nernst equation). Extrapolation of this inference defines $\log(PI_{ABS})$ as the driving force (DF_{ABS}) for photosynthesis of the observed system, created by summing the partial driving forces for each of the several energy bifurcations (all at the onset of the fluorescence rise O-J-I-P):

$$DF_{ABS} = \log(PI_{ABS}) = \log[RC/ABS] + \log[\varphi_{Po}/(1 - \varphi_{Po})] + \log[\Psi_o/(1 - \Psi_o)]$$

Should $DF_{RC} = [RC/ABS]$, $DF_\varphi = \log[\varphi_{Po}/(1 - \varphi_{Po})]$ and $DF_\Psi = \log[\Psi_o/(1 - \Psi_o)]$, then the above equation can be written as:

$$DF_{ABS} = DF_{RC} + DF_\varphi + DF_\Psi.$$

The JIP-test reveals changes in PSII behaviour that the commonly used $\varphi_{Po} = (F_M - F_0)/F_M$ is not able to detect, and since φ_{Eo} and PI are related to the productivity of photosynthetic metabolites, they offer a diagnostic tool for biomass production capability (Strasser *et al.*, 2001).

3.2.2. Results and Discussion:

3.2.2.1. Effect of TCA on the processes of primary photochemistry:

3.2.2.1.1. *Phaseolus vulgaris*:

At low concentrations TCA seemed to have had a stimulatory effect on *Phaseolus vulgaris*, while higher concentrations had an inhibitory effect on certain metabolic functions. This phenomenon was reflected by most of the fluorescence parameters determined by means of the JIP-test; thus certain parameters showed an initial increase with TCA-treatment, followed by a decrease at higher TCA-concentrations. This observation is confirmed by the findings in 3.1.2.1.1. and 3.3.2.1.1., where CO_2 assimilation and Rubisco activity data showed initial stimulation at lower levels of TCA treatment, but inhibition at higher TCA concentrations.

The performance index calculated on absorption basis (PI_{ABS}) proved to be a sensitive indicator of the effect of TCA (Figure 3.20). Although the difference in PI_{ABS} of the control plants and TCA-treatments of 0.05 and 0.20 g/m^2 was not statistically significant, higher concentrations of 0.80 and 3.20 g/m^2 caused decreases of 19% and 51% respectively ($p<0.01$ in both cases). The same trend could be observed in the driving force values (DF = $\log PI_{ABS}$), with no significant changes in the lower TCA-concentrations, but decreases with treatments of 0.80 and 3.20 g/m^2.

The fluorescence data revealed that the different partial processes of primary photochemistry i.e. the partial driving forces of light absorption, trapping and conversion of excitation energy to electron transport, were differentially affected by the TCA treatment (Figure 3.21). The partial driving force of absorption flux (logRC/ABS) decreased by up to 16% at a TCA-concentration of 3.20 g/m^2 ($p<0.01$), as was the case with the partial driving force of trapping ($\log\varphi_{Po}/1-\varphi_{Po}$), with a decrease of up to 17% ($p<0.01$). The partial driving force of conversion of excitation energy to electron transport ($\log\psi_o/1-\psi_o$) increased with 21% relative to the control at 0.05 g TCA/m^2. In the case of the 0.20 g TCA/m^2 treatment an increase of 9% occurred at in this parameter. Higher concentrations of TCA however led to a decrease in $\log\psi_o/(1-\psi_o)$, namely 4% and 30% at 0.80 and 3.20 g TCA/m^2 respectively, of which only the aforementioned decrease was significant ($p<0.01$).

The changes in the different parameters of primary photochemistry of PSII, as calculated by the formulae shown in Table 3.5, are demonstrated in Figure 3.22 (a) to (c).

At the highest TCA concentration of 3.20 g/m^2, the turnover number (N) increased with 22% ($p<0.01$) (Figure 3.22a). This is corroborated by the 29% decrease in $(1 - V_J)/V_J$ ($p<0.01$) (Figure 3.22c), which is an indication of a decrease in efficiency to convert excitation energy to electron transport beyond Q_A. A lower concentration of 0.05 g TCA/m^2 caused an initial decrease of 10% ($p<0.01$) in V_J, after which it started to increase with increasing TCA dosage, to 13% ($p<0.01$) above the control values at 3.20 g TCA/m^2 (Figure 3.22a). The initial

decrease in V_J is an indication of a decrease in Q_A^- pool size, which is a sign of increased metabolism. Thus metabolism was stimulated by the lower TCA concentrations, while the higher concentrations caused a decrease in metabolism, since the increase in V_J indicates an increase in Q_A^- pool size, which represents a lowered metabolism.

With respect to the specific energy fluxes, the following changes occurred during the TCA-treatments: (i) lower concentrations of TCA caused only small increases in ABS/RC (antenna size), while a very high dosage of 3.20 g/m^2 caused an increase of 16%; (ii) the same observation was true for TR_0/RC, showing an increase of 12% at 3.20g TCA/m^2; (iii) in the case of ET_0/RC, there was an increase of 16% at the lowest TCA-treatment of 0.05 g/m^2 ($p<0.01$), after which a decrease could be observed, with the values falling to 8% of the control at 3.20g TCA/m^2 ($p<0.01$).

The increase in antenna size (ABS/RC) and specific trapping flux (TR/RC) coincided with a decrease in density of reaction centres (RC/CS) (Figure 3.22b) which resulted in a very small decrease in the quantum efficiency of primary photochemistry (φ_{P0}). The large decrease in electron transport per cross section (ET/CS) at the higher TCA concentrations, must mainly be ascribed to the reduction in density of RC's (Figure 3.22b) and the decrease in the efficiency of conversion of excitation energy to ET beyond Q_A ($1-V_J/V_J$) (Figure 3.22c).

Changes in the phenomenological energy fluxes were similar to those found in the specific energy fluxes. Low concentrations of TCA had little, although significant ($p<0.05$), effect on ABS/CS, with a concentration of 3.20g TCA/m^2 causing a 13% increase ($p<0.01$). The TR_0/CS also showed only small, although significant ($p<0.05$), changes with lower TCA-concentration, with an increase of 10% ($p<0.01$) at a concentration of 3.20 g/m^2. An initial increase of 11% occurred in ET_0/CS at a low concentration of 0.05 g TCA/m^2, after which it decreased with higher concentrations down to 11% of the control value at 3.20 g TCA/m^2, as was the case with the energy fluxes per RC.

There were significant changes ($p<0.01$) in the number of active RCs per cross-section of leaf, with the density of active RCs decreasing as the TCA dosage was increased (Figure 3.22b).

3.2.2.1.2. *Zea mays*:

TCA-treatment seemed to have had a similar inhibitory effect on *Zea mays* as on *Phaseolus vulgaris*, with the plants exhibiting an initial stimulation in growth at lower TCA concentrations, followed by a decline in growth at higher concentrations. The first evidence of this phenomenon is found in the value of the performance index (Figure 3.23), which increased by 7% ($p<0.05$) at the low concentration of 0.05 g TCA/m^2, and then started to decrease with

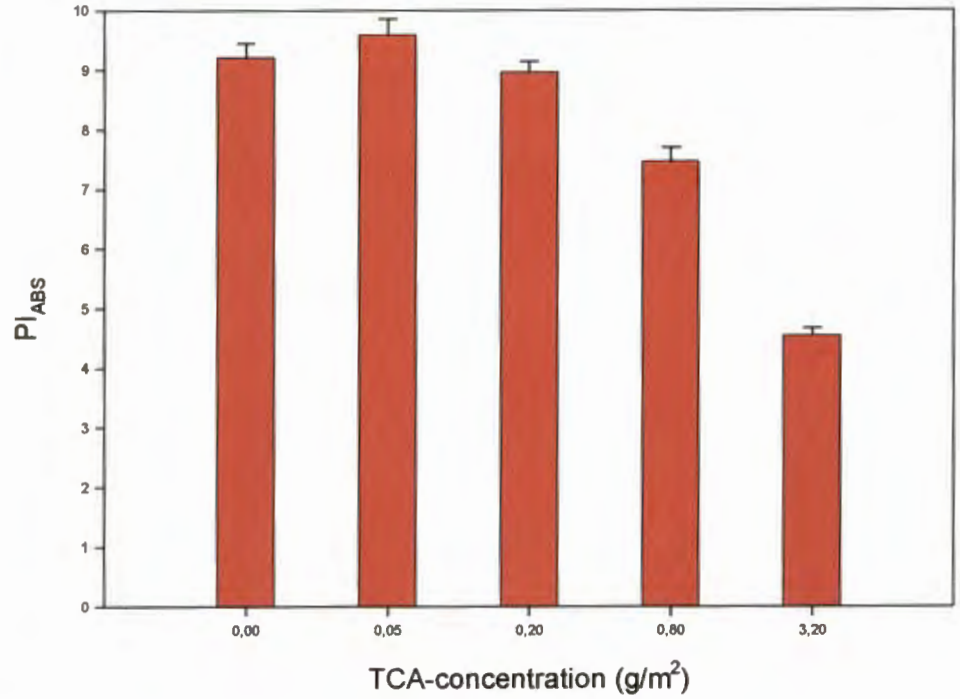


Figure 3.20 Performance index (expressed on absorption basis, PI_{ABS}) of *Phaseolus vulgaris* as a result of exposure to different TCA-concentrations.

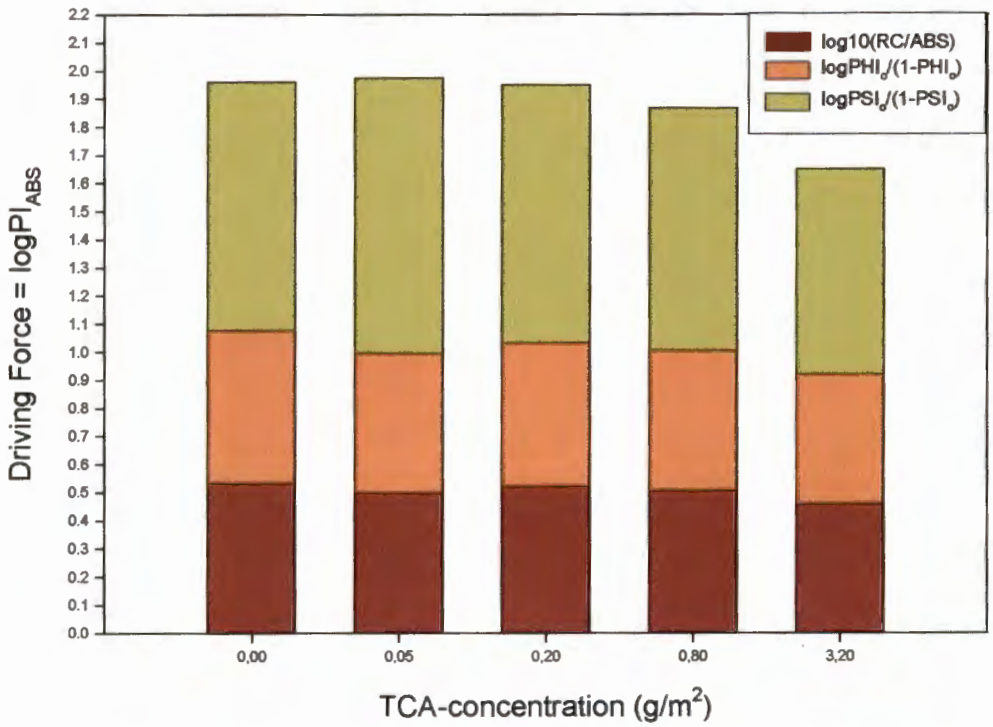


Figure 3.21 Visualisation of the relative changes in the partial driving forces of primary photosynthesis in *Phaseolus vulgaris* after exposure to a range of TCA-concentrations.

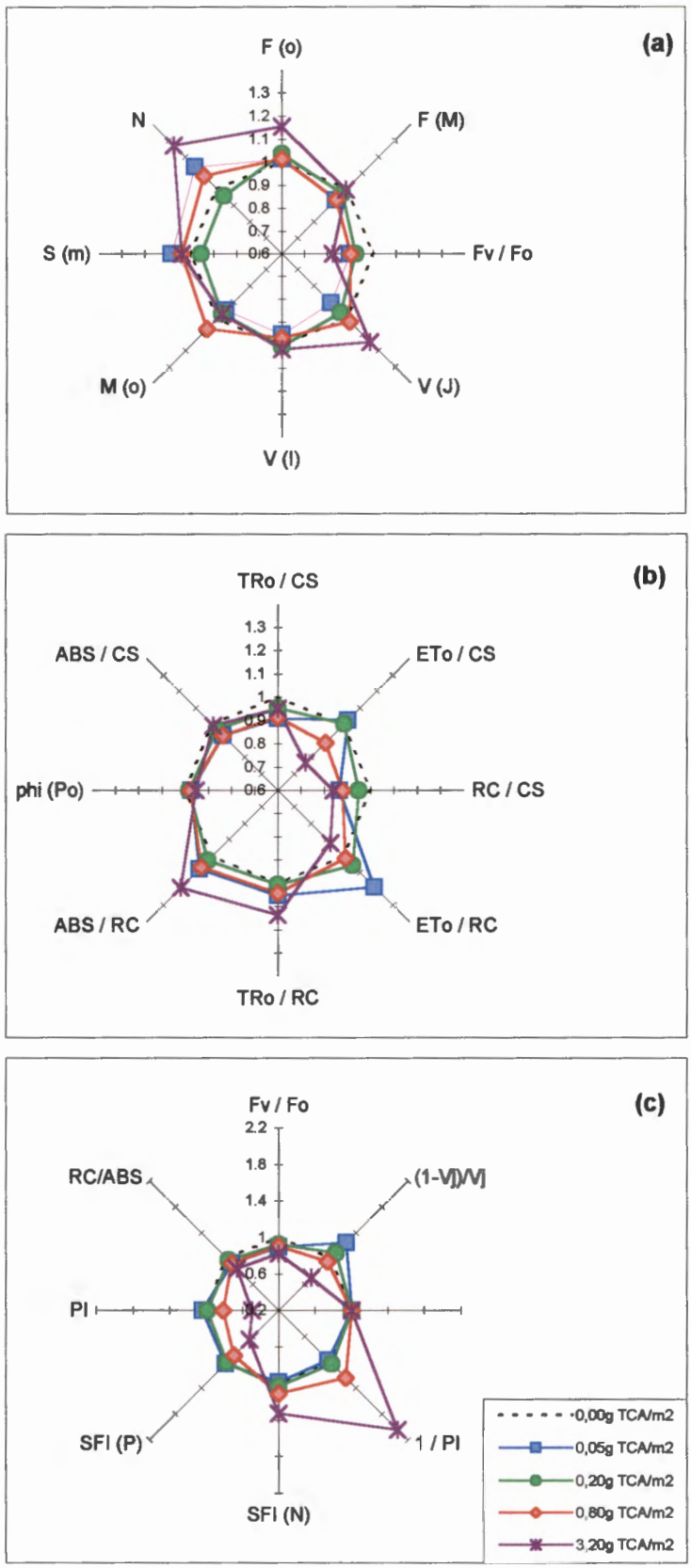


Figure 3.22 Changes in the technical (a), functional (b) and vitality/efficiency parameters (c) of primary photochemistry in *Phaseolus vulgaris* after exposure to TCA at concentrations of 0.05, 0.20, 0.80 and 3.20 g/m^2.

higher treatments, namely 18% at 0.20, 37% at 0.80 and 68% at 3.20 g TCA/m^2 ($p<0.01$ in all cases).

The logarithm of the PI_{ABS} (the driving force), indicates a similar trend (Figure 3.24). If the partial driving forces are taken into consideration, it is clear that the parameter affected most and thus responsible for the change in the total driving force, is $\psi_0/(1-\psi_0)$. $\psi_0/(1-\psi_0)$ showed an initial 10% increase ($p<0.01$) at 0.05 g TCA/m^2, followed by decreases of 16%, 17% and 45% at the higher concentrations of 0.20, 0.80 and 3.20 g TCA/m^2 respectively ($p<0.01$ in all cases). The other contributing partial driving forces, RC/ABS and $\varphi_{P0}/(1-\varphi_{P0})$, remained relatively constant at lower TCA-concentrations, decreasing only at 0.80 g TCA/m^2 with 13% and 14% respectively, and with 18% and 28% respectively at 3.20 g TCA/m^2 ($p<0.01$ in all cases).

In Figure 3.25 (a) to (c) the effect of the different TCA-treatments on each of the parameters of primary photochemistry is demonstrated. Among the functional parameters, there was an increase of 20% ($p<0.01$) in the turnover number (N) at the highest TCA concentration used (Figure 3.25a). This corresponds to the large decrease of 45% ($p<0.01$) found in $(1 - V_J)/V_J$ (Figure 3.25c), indicating a decrease in the efficiency of conversion of excitation energy to electron transport beyond Q_A. An initial decrease of 6% ($p<0.01$) occurred in V_J at 0.05 g TCA/m^2, hereafter increasing TCA dosages resulted in an increase in V_J, with the values being 26% ($p<0.01$) above the control values at 3.20g TCA/m^2. The initial decrease in V_J was the result of a decrease in Q_A^- pool size, which is an indication of increased metabolism, while the subsequent increase in V_J points to an increase in Q_A^- pool size, and thus inhibited metabolism.

Among the functional parameters representing the specific energy fluxes, ABS/RC (antenna size) showed little change at lower TCA concentrations, while increases of 13% ($p<0.01$) and 18% ($p<0.01$) occurred at higher concentrations of 0.80 and 3.20 g TCA/m^2 respectively (Figure 3.25b). TR_0/RC also increased with 9% and 10% at these TCA-dosages ($p<0.01$ in both cases), remaining constant at lower levels of TCA-exposure. ET_0/RC showed changes in accordance with the observation of an initial stimulation, followed by an increasing inhibition at higher concentrations: at 0.05 g TCA/m^2 the electron transport per RC increased by 6% ($p<0.01$), and then decreased to 16% of the control ($p<0.01$) at a higher TCA-concentration of 3.20 g/m^2.

On the phenomenological level, TR_0/CS gradually decreased, while the ABS/CS increased by only 8% at a TCA-concentration of 3.20 g/m^2 ($p<0.01$). Similar to the energy flux per reaction centre, the electron transport per cross section of the leaf (ET_0/CS) showed an initial rise of 7% at 0.05 g TCA/m^2 ($p<0.01$), followed by a decrease down to 25% of the control at 3.20g TCA/m^2 ($p<0.01$). The effect of TCA on the electron transport per cross section was mainly

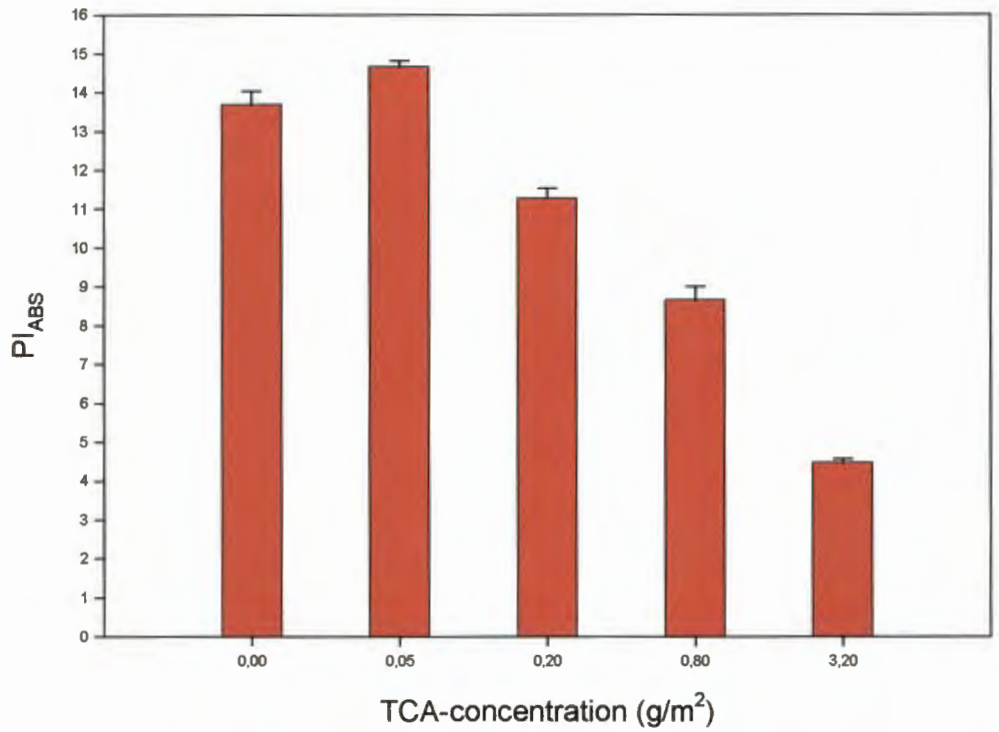


Figure 3.23 Change in the performance index (expressed on absorption basis) of *Zea mays* as a result of treatment with different concentrations of TCA.

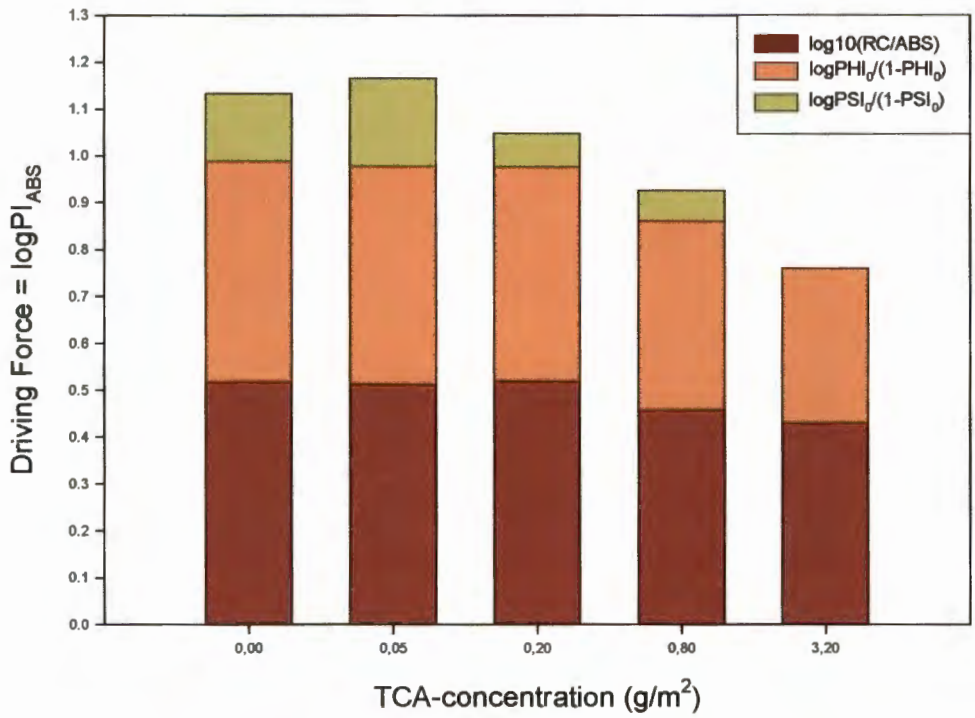


Figure 3.24 Visualisation of the relative changes in the partial driving forces of photosynthesis in *Zea mays* after exposure to different concentrations of TCA, compared to the control.

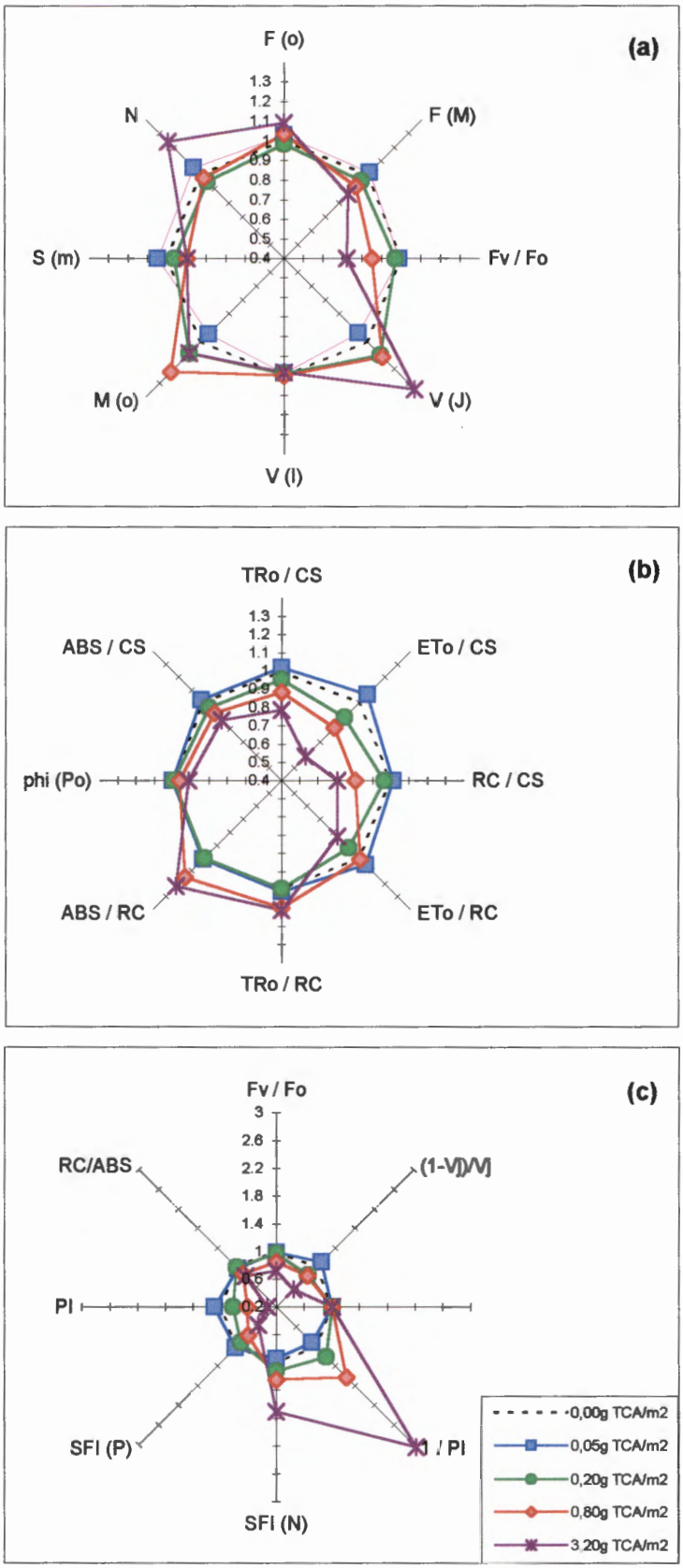


Figure 3.25 Changes in the technical (a), functional (b) and vitality/efficiency parameters (c) of primary photochemistry in *Zea mays* after exposure to TCA at concentrations of 0.05, 0.20, 0.80 and 3.20 g/m^2.

due to the changes in the density of reaction centres (RC/CS) (Figure 3.25b). At the lowest TCA concentration of 0.05 g/m^2, an increase in RC/CS occurred leading to an increase in ET_0/CS. Thereafter increasing TCA treatments lead to reduction in RC/CS (10% at 0.80 and 3.20 g TCA/m^2, with $p<0.01$ in both cases) which was reflected in a concomitant decrease in ET_0/CS. The fact that only a moderate corresponding change in φ_{P0} took place, was due to the concurrent increase in antenna size (ABS/RC) and the specific trapping flux (TR/RC) which compensated for the decrease in RC/CS (Figure 3.25b). These results accentuate the powerful ability of the JIP test to convolute the fluorescence signal into different energy fluxes (Krüger *et al.*, 1997). In many studies $\varphi_{P0} = F_V/F_M$, with its restricted sensitivity, is the only fluorescence parameter used. Figure 3.25c depicts the relationship between the concentration of the TCA treatment and the PI value, indicating that the decrease in PI (increase in 1/PI) is mainly due to the decreased efficiency of converting excitation energy to electron transport beyond Q_A (See also Figure 3.24).

3.2.2.2. Effect of TECE on the processes of primary photochemistry:

3.2.2.2.1. *Phaseolus vulgaris*:

When only the values of PI_{ABS} are taken into account, the effect of TECE on *Phaseolus vulgaris* after one week of exposure does not seem to be significant (Figure 3.26). If however the different partial driving forces constituting the Driving Force are compared, significant differences come to light (Figure 3.27). Of the three key steps of primary photochemistry, the partial driving force of trapping ($\log\varphi_{P0}/1-\varphi_{P0}$) was not affected, while the partial driving forces of absorption flux (logRC/ABS) and conversion of trapped excitation energy to electron transport ($\log\psi_0/1-\psi_0$) both showed decreases of 11% ($p<0.01$ in both cases) with TECE-treatment.

Figure 3.28 (a) to (c) demonstrates the change in each of the parameters of primary photochemistry of PSII, as calculated by the formulae shown in Table 3.5. The functional parameter V_J increased with 4% ($p<0.05$) as a result of exposure to TECE vapour, indicating a decrease in metabolism due to an increase in the pool size of Q_A^- (Figure 3.28a). The maximum rate of primary photochemistry (M_0 or dV/dt_0) decreased by 8% ($p<0.01$), corresponding with the 11% ($p<0.01$) decrease in maximum trapping flux (TR_0/RC) (Figure 3.28b).

Changes in the specific (per RC) and phenomenological (per leaf CS) energy fluxes were observed (Figure 3.28b). Exposure of the test plants to TECE vapour caused a decrease in all the specific energy fluxes: ABS/RC (antenna size), TR_0/RC (specific trapping flux) and ET_0/RC ($p<0.01$ for all), as well as the phenomenological energy fluxes: ABS/CS, TR_0/CS and ET_0/CS ($p<0.01$ for all). The maximum quantum yield of primary photochemistry (φ_{P0}) however showed almost no change (Figure 3.28b). Thus, although according to the PI_{ABS}-value no apparent changes occurred in the photochemical mechanisms of *P. vulgaris* as a

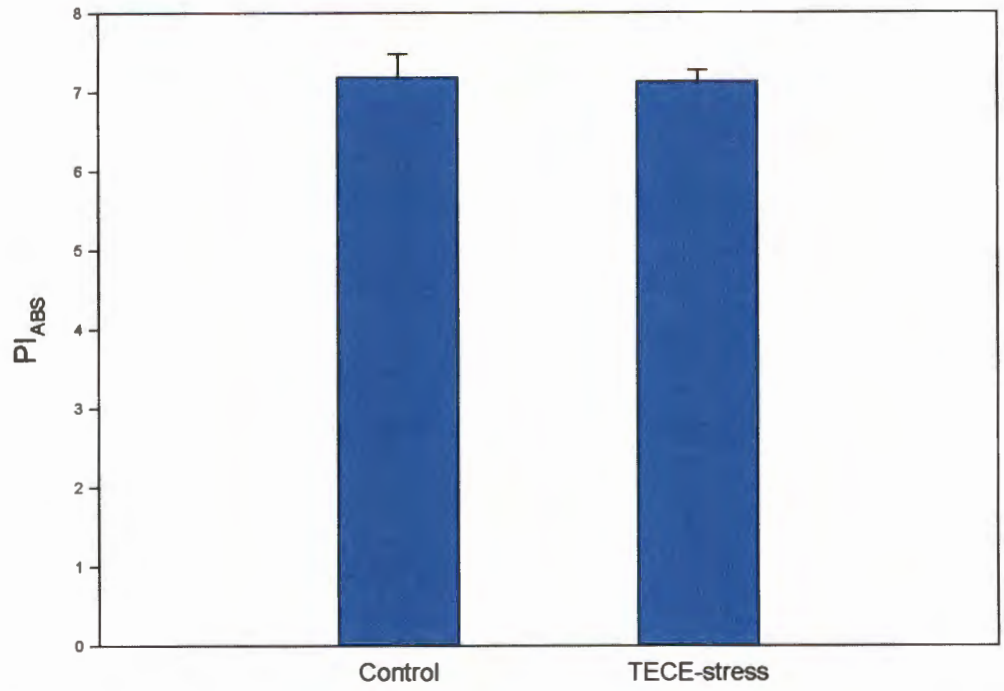


Figure 3.26 Effect of exposing *Phaseolus vulgaris* plants for one week to TECE vapour on the vitality of the plants expressed by PI_{ABS} values.

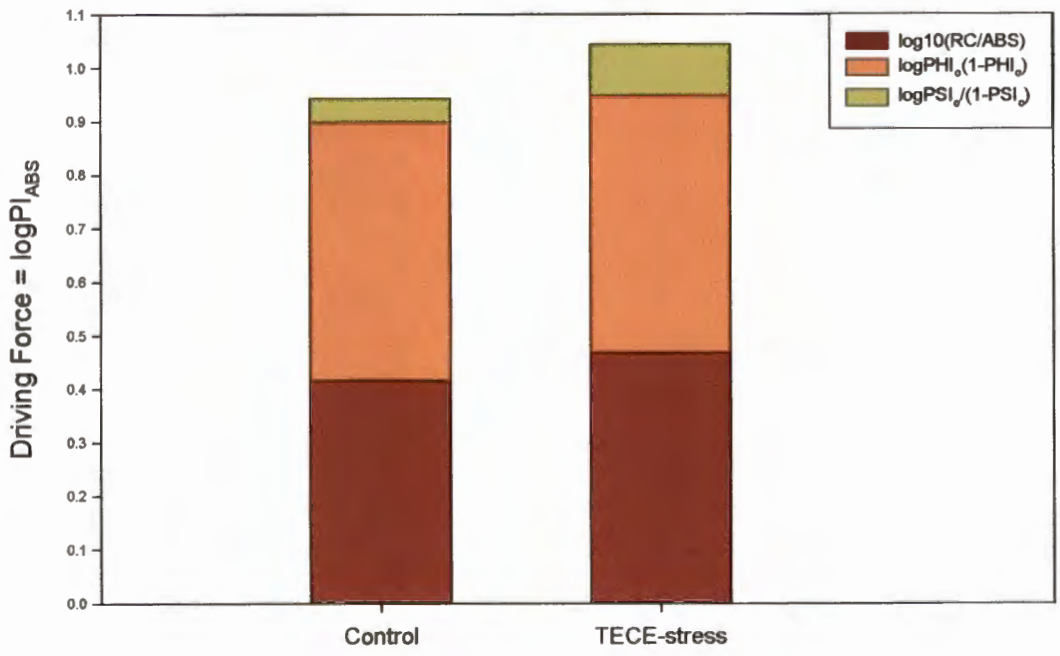


Figure 3.27 Visualisation of the relative changes in the partial driving forces of primary photosynthesis in *Phaseolus vulgaris* after one week of exposure to TECE vapour.

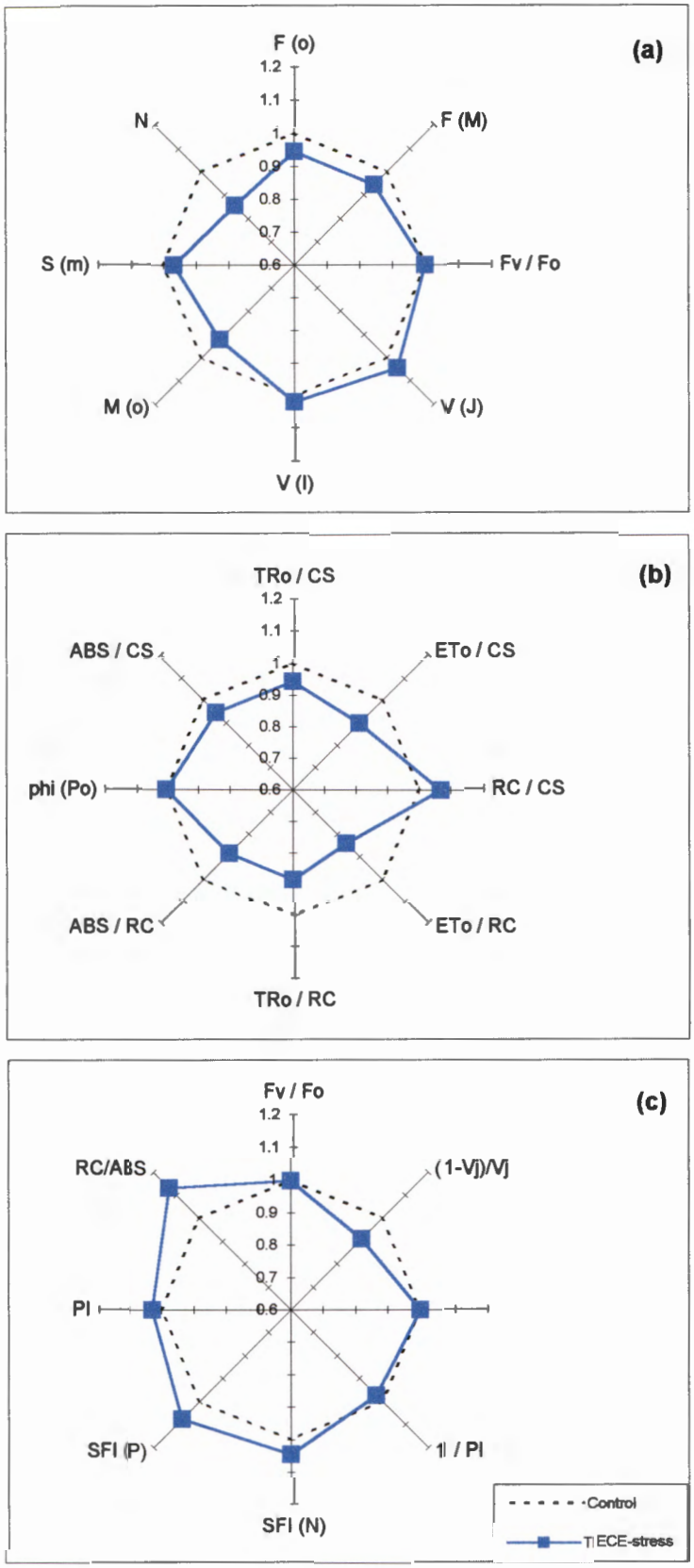


Figure 3.28 Changes in the technical (a), functional (b) and vitality/efficiency parameters (c) of primary photochemistry in *Phaseolus vulgaris* after one week of exposure to TECE at a concentration of 810 g/m^3.

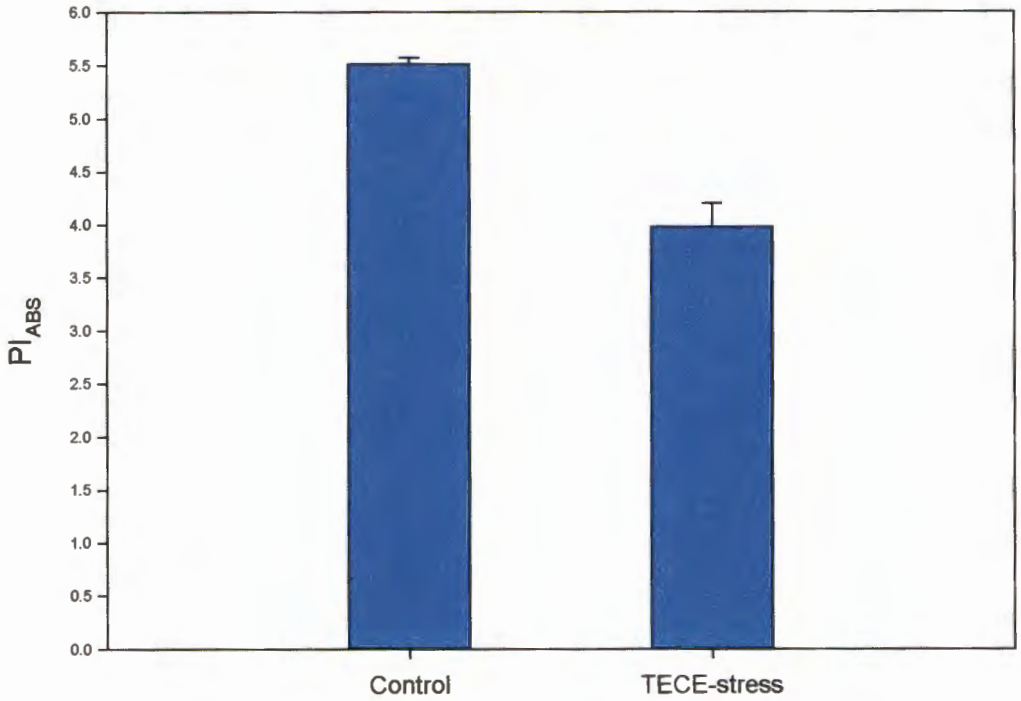


Figure 3.29 Effect of exposing *Zea mays* plants for one week to TECE vapour on the vitality of the plants expressed by PI_{ABS} values.

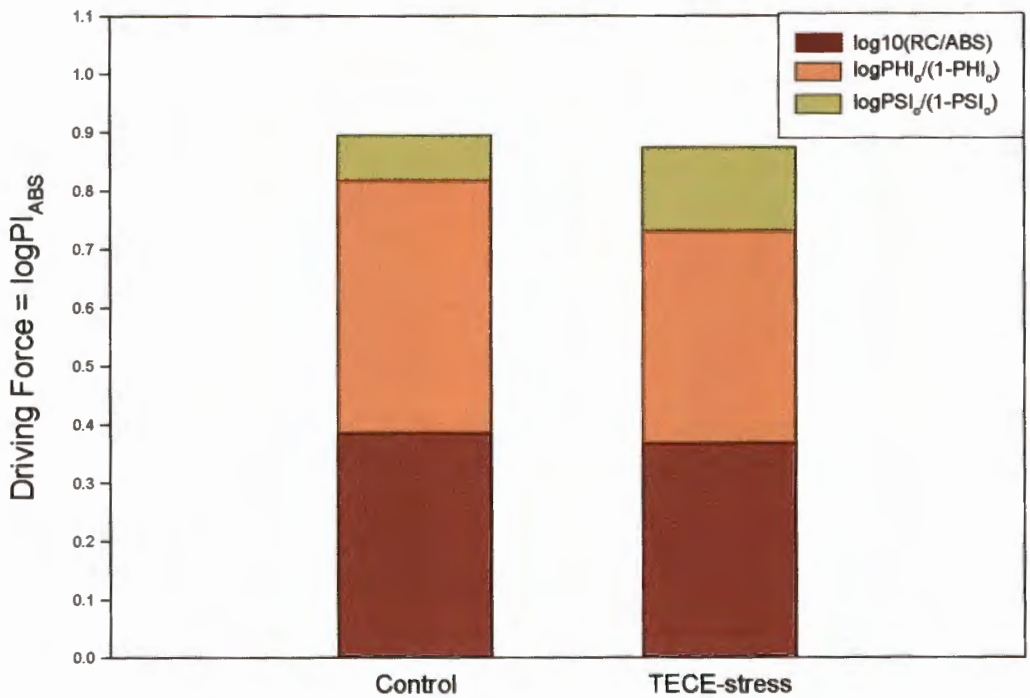


Figure 3.30 Visualisation of the relative changes in the partial driving forces of photosynthesis in *Zea mays* after one week of exposure to TECE vapour, compared to the control.

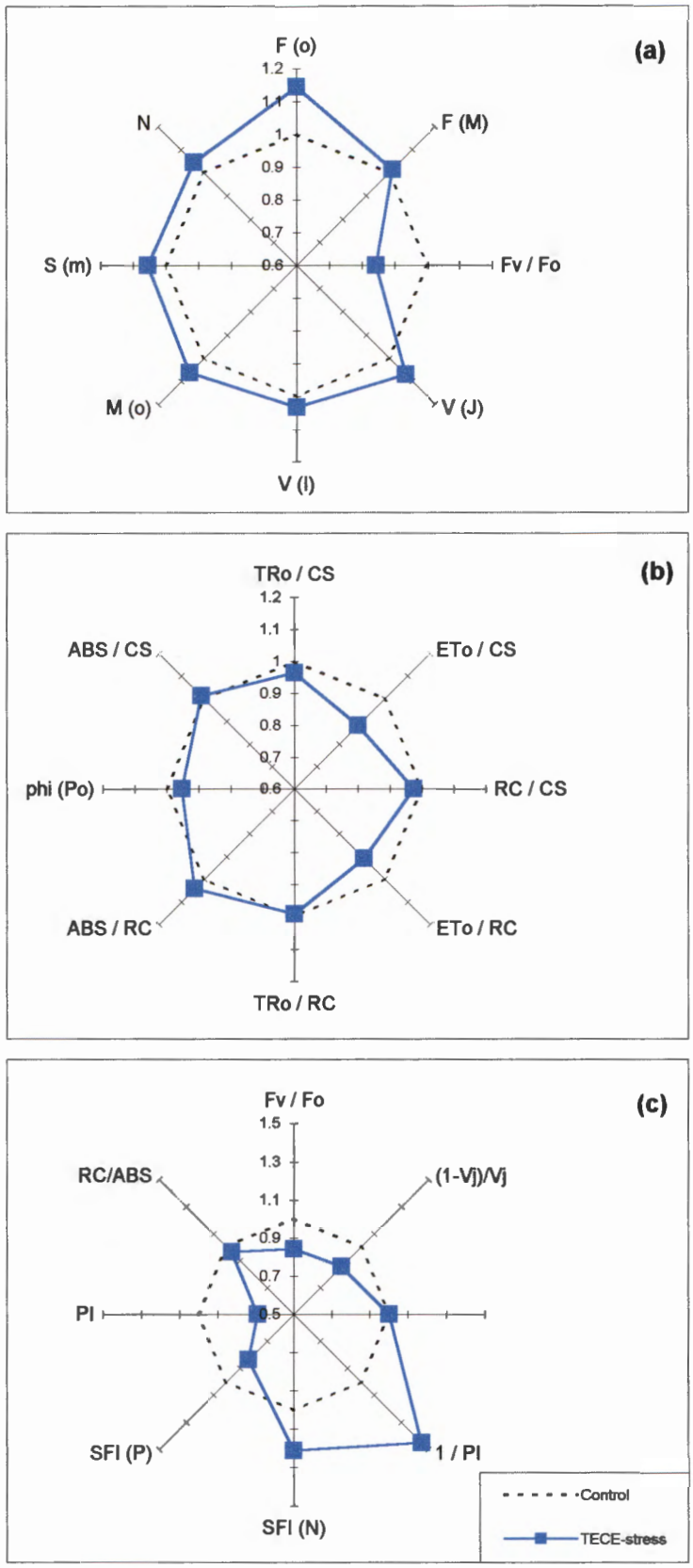


Figure 3.31 Spider plots depicting the changes in the technical (a) functional (b) and vitality/efficiency parameters (c) in *Zea mays* after one week exposure to TECE vapour.

result of exposure to TECE vapour, further analysis of the effect on the different contributing parameters revealed significant changes, i.e. decreases in absorption flux and electron transport.

3.2.2.2.2. *Zea mays*:

The PI_{ABS} of *Zea mays* decreased with 28% ($p<0.01$) after a week of exposure to TECE (Figure 3.29). This was as a result of decreases in all three the partial driving forces of primary photochemistry: logRC/ABS ($p<0.05$), $\log\varphi_{P0}/(1-\varphi_{P0})$ ($p<0.01$) and $\log\psi_0/(1-\psi_0)$ ($p<0.01$). In the case of logRC/ABS, the TECE-treatment caused only a 4% decrease, while $\log\varphi_{P0}/(1-\varphi_{P0})$ and $\log\psi_0/(1-\psi_0)$ showed larger decreases of 15% and 14% respectively (Figure 3.30). These findings are corroborated by Figure 3.31c, which shows an increase in 1/PI, and thus a decrease in PI as a result of exposure to TECE vapour.

Figure 3.31 (a) to (c) demonstrates the changes in each of the parameters of primary photochemistry. The intensity of fluorescence at 50 µs (F_0) was increased by 20% ($p<0.01$) as a result of the exposure to TECE. However, the maximal fluorescence intensity (F_M) showed no significant change. The 7% ($p<0.01$) increase in V_J points to an increase in the pool size of Q_A^-, which is the result of a decrease in metabolism (Figure 3.31a).

Investigation of the specific energy fluxes revealed an increase in antenna size (ABS/RC) ($p<0.01$) and a decrease in ET_0/RC ($p<0.01$), while the maximum trapping flux (TR_0/RC) showed no change after TECE-treatment. In the case of the functional parameters of the phenomenological energy fluxes, ABS/CS showed insignificant change, with decreases of 4% ($p<0.05$) in TR_0/CS and 12% ($p<0.01$) in the electron transport per cross section (Figure 3.31b). ET_0/CS most probably decreased as a result of the decrease in efficiency to convert excitation energy to electron transport beyond Q_A ($1 - V_J/V_J$, Figure 3.31c).

The maximum quantum yield of photochemistry, φ_{P0}, decreased significantly with 5% ($p<0.01$), but this change did not have an effect on RC/CS. This could explain the small increase in antenna size (ABS/RC). This is also corroborated by the small change in F_M (Figure 3.31a), since an increase in this parameter is consistent with closure of RCs (Figure 3.31b).

3.3. Rubisco-activity:

3.3.1. Rubisco: Function and mechanism:

The enzyme rubilose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) is the most abundant enzyme on earth, consisting up to half of the total leaf protein in C_3 plants. It catalyses the first phase of the Calvin cycle namely carboxylation (Leegood, 1993):

$$RuBP + CO_2 + H_2O \xrightarrow{\text{Rubisco}} 2(\text{3-phosphoglycerate}) + 2H^+$$

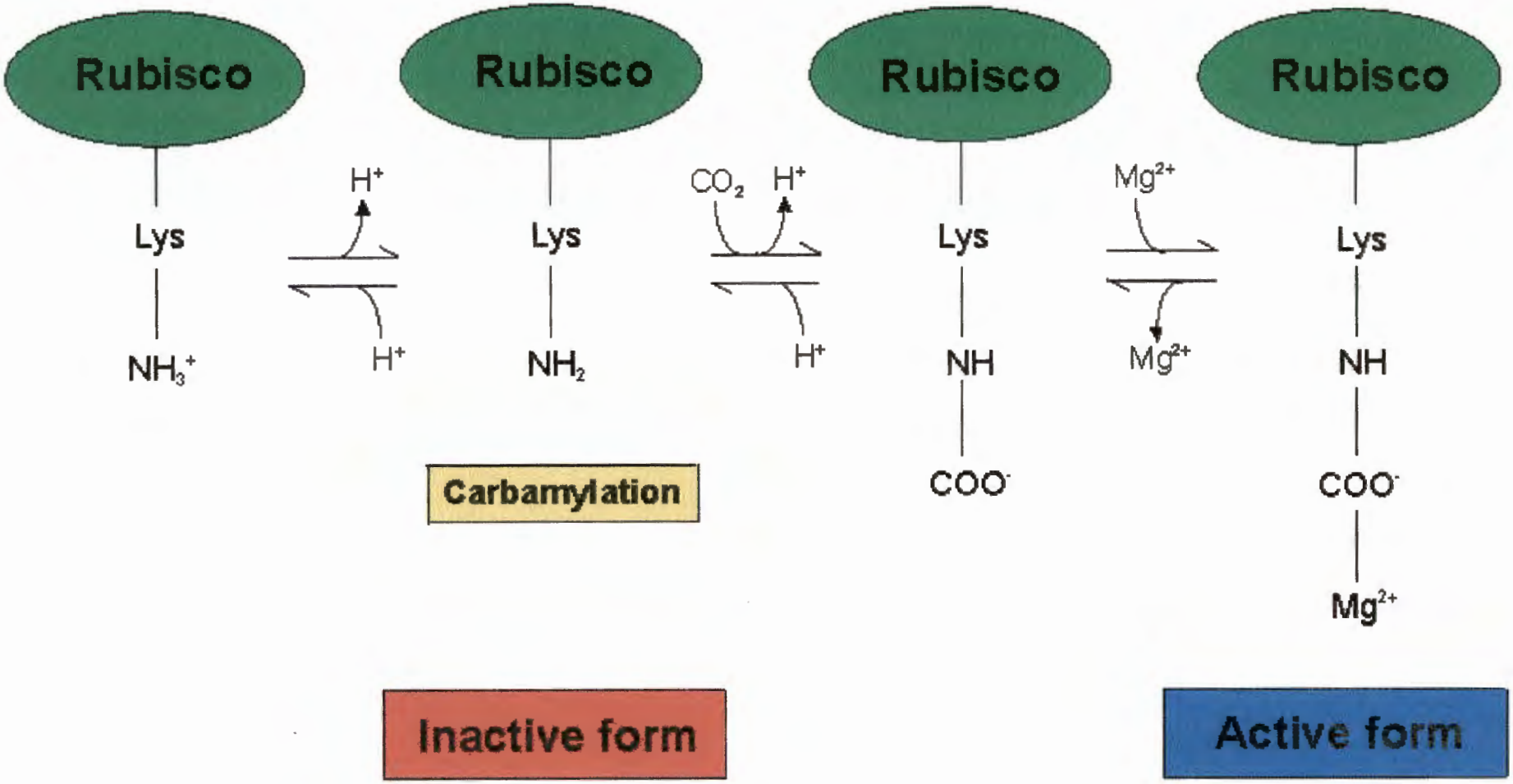


Figure 3.32 Rubisco is activated when a carbamate-Mg^{2+} complex is formed on the ε-amino group of a lysine within the active site of the enzyme. Other than by an increase in Mg^{2+} concentrations, activation is also stimulated by an increase in pH, since two protons are released. Illumination would seem to be the reason for the increase in stromal pH and Mg^{2+} concentrations.

In C_4 plants photorespiration is suppressed as a result of the saturating supply of CO_2 to Rubisco, and because C_4 plants possess an ATP-dependant CO_2 pump which is closely co-ordinated with the Calvin cycle (Leegood, 1993).

Rubisco consists of eight large and eight small subunits (L_8S_8) and shows light-induced transitions to active forms (Leegood, 1993). Assembly of the subunits is achieved by coordinated biochemical processes occurring in several cellular compartments. Light modulates expression of the gene rbcS in the nuclear genome, producing a precursor polypeptide of the small subunit which is translated by cytosolic ribosomes. The gene rbcL, situated in the chloroplast genome, codes for the mature form of the large subunit, which is synthesised on chloroplast ribosomes. Rubisco is finally assembled from its constituent subunits in the chloroplast, assisted by special modulating proteins called chaperonins (Buchanan and Wolosiuk, 1998).

Rubisco is a bifunctional enzyme, catalysing incorporation of both O_2 and CO_2: The oxygenation acts as a competitive inhibitor of carboxylation and thereby provides the glycollate for the photorespiratory pathway (Ogren, 1984). Rubisco is subject to two forms of regulation. By reacting firstly with CO_2 and then Mg^{2+}, it is converted to an active carbamylated form. The ATP-dependant enzyme Rubisco activase ensures activation *in vivo* where CO_2 concentrations are too low for adequate activation *in vitro* (Salvucci, 1989). It also reactivates Rubisco that has been deactivated by sugar bisphosphates by accelerating the release of the inhibited decarbamylated form (Salvucci and Ogran, 1996). In some cases preincubation with CO_2 and Mg^{2+} is insufficient for full activation of Rubisco. This usually occurs at night and low light, when some plants synthesise a natural tight-binding competitive inhibitor, namely carboxyarabinitol-1-P (Salvucci, 1989). Rubisco is activated when activator CO_2 (not substrate CO_2) reacts with an uncharged ε-NH_2 group of lysine on the active site of the enzyme. A carbamate derivative is then formed which binds with Mg^{2+} to form the activated complex. Thus light-activation of Rubisco seems to be mediated by light-dependant stromal changes in Mg^{2+} and pH, since two protons are released (Lorimer, 1981) (Figure 3.32).

3.3.2. Results and Discussion:

3.3.2.1. Effect of TCA on the activity of Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco):

3.3.2.1.1. *Phaseolus vulgaris*:

The only significant change in the initial specific activity of Rubisco in *P. vulgaris* treated with TCA (Figure 3.33a) was a 23% decrease at 0.20 g TCA/m^2 ($p<0.05$). The increase of 4% at 0.05 g TCA/m^2, as well as the subsequent increases of 20% and 6% at 0.80 and 3.20 g TCA/m^2, respectively, was not statistically significant. The maximum specific activity (after removal of tight-binding inhibitors) of Rubisco (Figure 3.33a) decreased by 32% ($p<0.05$),

53% ($p<0.01$) and 46% ($p<0.01$) at 0.05, 0.20 and 0.80 g TCA/m^2 respectively, after which the activity increased to 10% above the control value at 3.20 g TCA/m^2. However, the large increase in maximum specific Rubisco activity at 3.20 g TCA/m^2 was most probably an artifact introduced by the large decrease in total soluble protein content at this concentration (Figure 3.34a). Although an initial increase in the total soluble protein content (on a leaf area basis) occurred at low TCA concentrations, the total protein content at 3.20 g TCA/m^2 was 15% lower than that of the control (Figure 3.34a). A very similar change in total chlorophyll content (on a leaf area basis) to that of soluble protein content was also observed at the different TCA concentrations (Figure 3.34b). Total chlorophyll content increased by 31% at 0.05 g TCA/m^2 ($p<0.01$), after which it gradually decreased to only 1% of the control values at 3.20 g TCA/m^2. This initial increase in total chlorophyll content at low TCA concentrations, with a subsequent decrease at higher TCA concentrations supports the findings reported in section 3.2.2.1.1., as revealed by chlorophyll the fluorescence data.

The activation state of Rubisco, which is the initial specific activity expressed as a percentage of maximum specific activity, initially increased by 29% ($p<0.05$) at 0.05 g TCA/m^2, and then by a further 36% and 26% at 0.20 and 0.80 g TCA/m^2 respectively (Figure 3.33b). At 3.20 g TCA/m^2, however, the activation state decreased by 22% compared to the values of the untreated plants. This was mainly a result of the large increase in maximum specific activity relative to the initial specific activity that occurred at this specific TCA concentration (Figure 3.33a).

It is concluded that by expressing Rubisco activity on a soluble protein basis, especially at high TCA concentrations, a false impression of the effect of TCA on Rubisco activity might be obtained, mainly because of the large changes in protein content induced by the treatments. By expressing Rubisco activity on a total chlorophyll basis (Figure 3.33c), a better reflection of the enzyme's response to high concentrations of TCA is obtained. Expression of the data in this fashion revealed that initial Rubisco activity gradually decreased with an increase in TCA concentration: 21% ($p<0.05$), 29% ($p<0.01$), 34% ($p<0.01$) and 24% ($p<0.01$) at 0.05, 0.20, 0.80 and 3.20 g TCA/m^2 respectively. Considering the fact that the total chlorophyll content of the control and plants treated with 3.20 g TCA/m^2 was very similar, the large inhibition of initial Rubisco activity can be regarded as a typical effect induced by TCA.

By calculating the initial activity of Rubisco on a leaf area basis, the interference caused by changes in protein and chlorophyll content is largely excluded. However, by expressing Rubisco activity in this fashion, new sources of variation is also introduced, because differences in the extraction efficiency during the assay procedure, variation in leaf thickness etc. is not taken into account. On a leaf area basis, an initial increase in Rubisco activity of 11% at 0.05 g TCA/m^2 was revealed, after which the activity decreased to values 23% lower

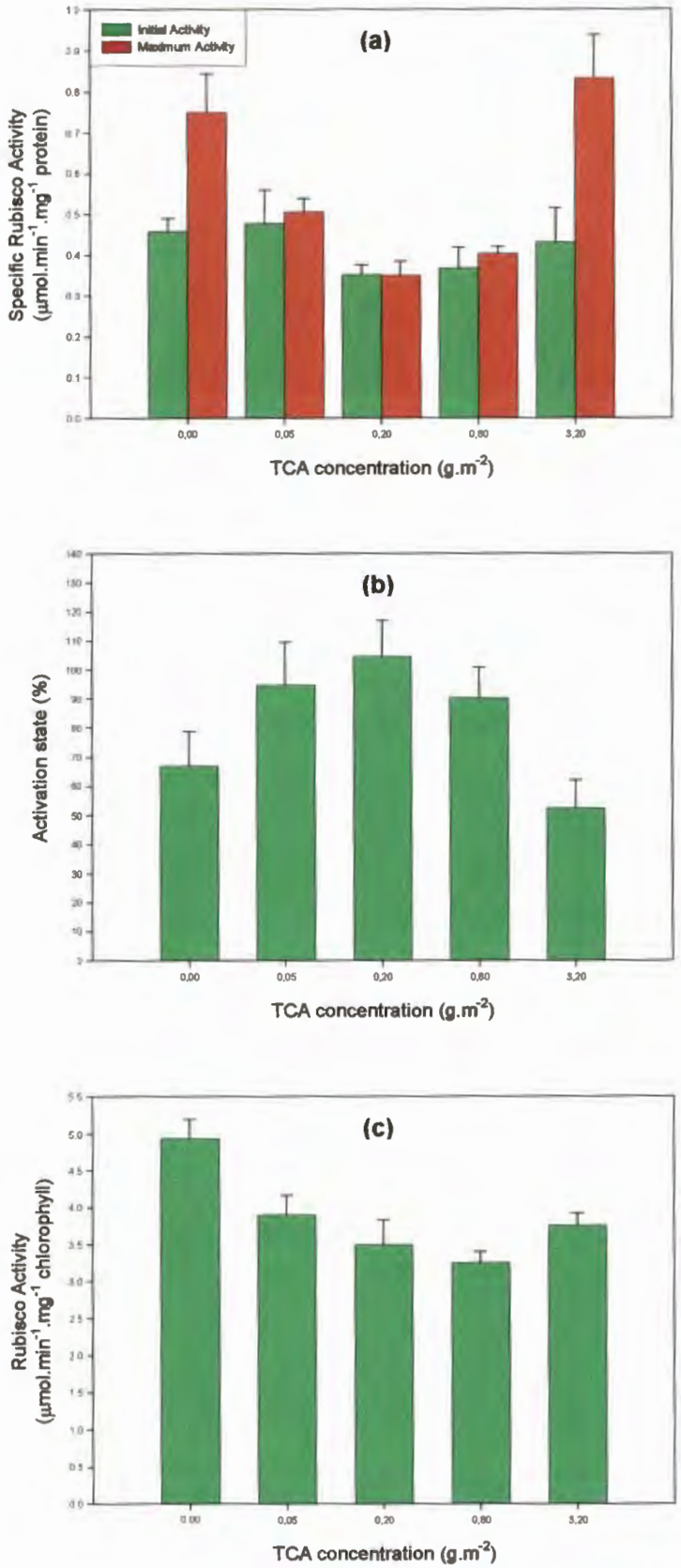


Figure 3.33 Effect of different TCA-concentrations on the initial and maximal rate of Rubisco activity (a), activation state (b) and Rubisco-activity expressed on a chlorophyll basis (c) in *Phaseolus vulgaris*. Each data point represents the mean of 4 to 6 replicates.

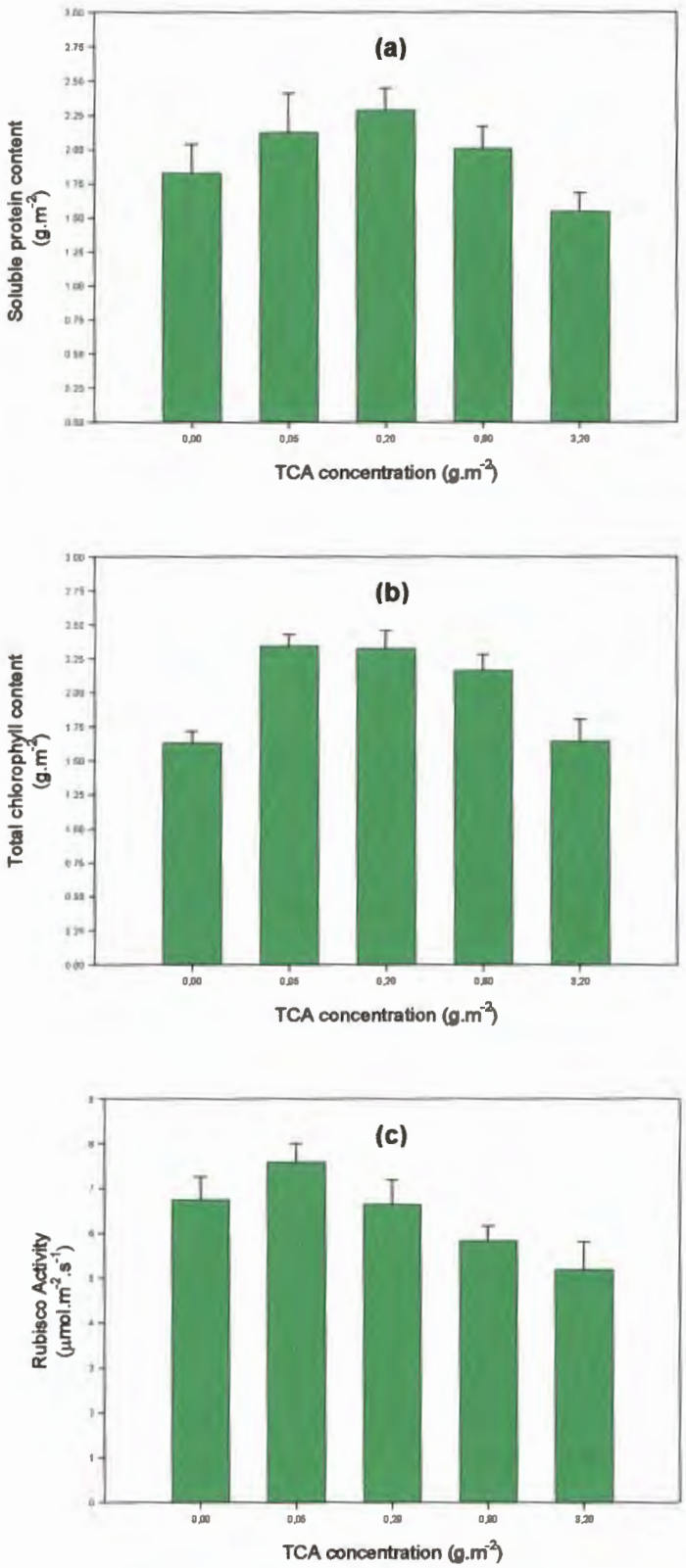


Figure 3.34 Effect of different TCA-concentrations on the total protein content per leaf area (a), total chlorophyll content per leaf area (b) and Rubisco-activity per sampled leaf disc (c) in *Phaseolus vulgaris*. Each data point represents the mean of 4 to 6 replicates.

than the control at 3.20 g TCA/m^2 (Figure 3.34c). None of these changes however were significant.

3.3.2.1.2. *Zea mays*:

In both the initial and maximum specific Rubisco activity a large decrease with increasing TCA concentration occurred, i.e. 81% ($p<0.01$) for initial and 62% for maximum specific activity (Figure 3.35a). When calculating the activation state of the enzyme (initial activity as a percentage of total activity), the only significant decrease (11%, $p<0.05$) appeared at 0.20 g TCA/m^2 (Figure 3.35b). The total soluble protein content per leaf area showed no significant change and no definite relationship was observed in response to the treatment with different TCA concentrations (Figure 3.36a). The total chlorophyll content per leaf area initially decreased by 22% ($p<0.05$) at 0.20 g TCA/m^2, and at a concentration of 3.20 g TCA/m^2, it decreased further to 29% ($p<0.01$) of the control (Figure 3.36b).

When expressing the data on a total chlorophyll basis, it was revealed that TCA treatment resulted in a decrease in initial Rubisco activity at most concentrations used. The degree of decrease was very similar at low TCA concentrations, while the highest concentration caused a considerable decrease. The values were 18%, 21% ($p<0.05$), 20% ($p<0.05$) and 73% ($p<0.01$) at 0.05, 0.20, 0.80 and 3.20 g TCA/m^2 respectively. The severe effect on Rubisco activity is further corroborated by the fact that the initial activity (expressed on a leaf area basis), also decreased in response to exposure to TCA. A significant decrease of between 39% and 77% occurred in response to an increase in TCA concentration between 0.20 and 3.20 g TCA/m^2 ($p<0.01$) (Figure 3.36c).

The change in protein content of the plants of different treatments differed less than the change in chlorophyll content. Thus the specific Rubisco activity should give a better indication than its activity expressed on chlorophyll basis. Although the chlorophyll content at a TCA concentration of 3.20 g/m^2 was very low, the Rubisco activity expressed on chlorophyll basis still showed a decrease. Normally a low chlorophyll content would cause Rubisco activity expressed on chlorophyll basis to be high. Nevertheless, in this case there was a decrease. This further supports the significant effect on Rubisco of *Zea mays* at high TCA concentrations.

3.3.2.2. Effect of TECE on the activity of Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco):

3.3.2.2.1. *Phaseolus vulgaris*:

The change in Rubisco activity of *Phaseolus vulgaris* after one week exposure to 810 µg TECE/m^3 was statistically significant, except when expressed on a leaf area basis (Figure 3.38c).

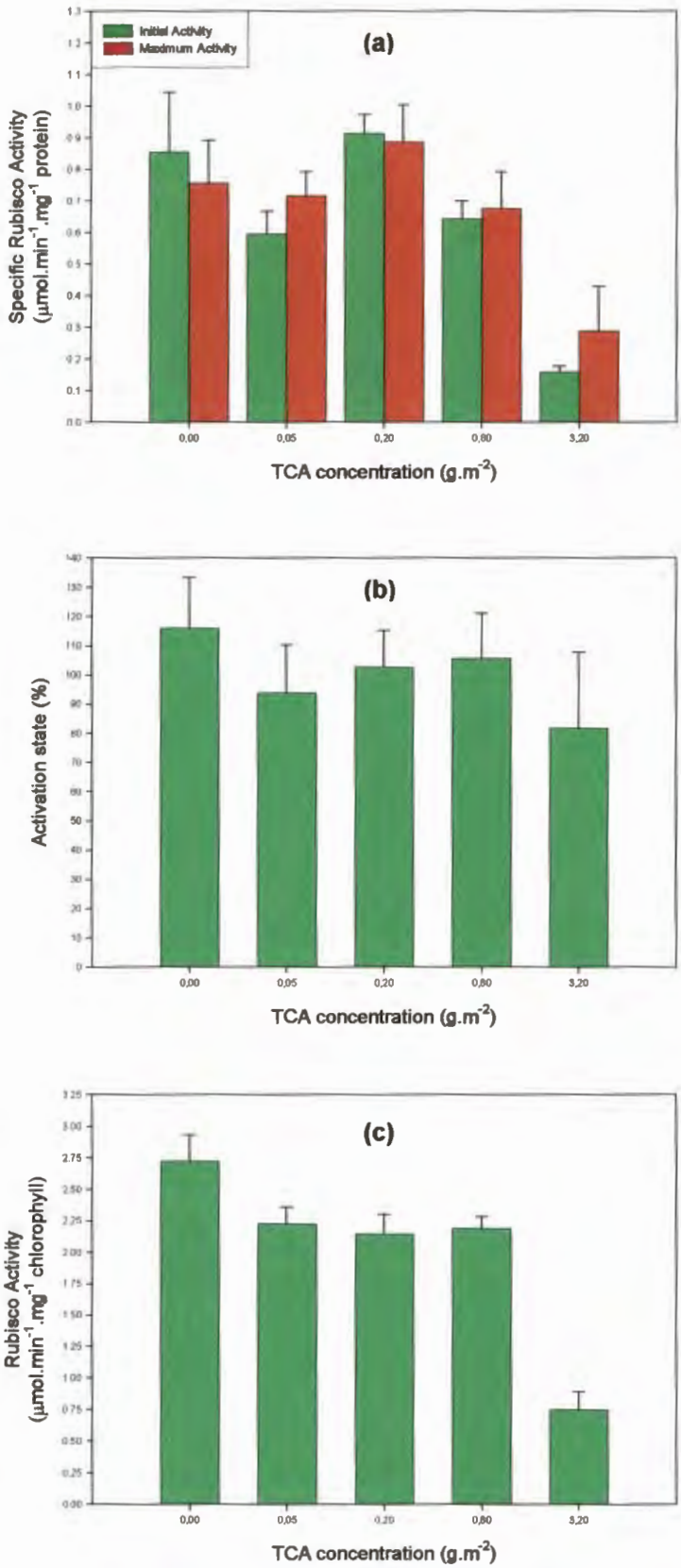


Figure 3.35 Effect of different TCA-concentrations on the initial and maximal activity rate of Rubisco (a), activation state (b) and Rubisco-activity expressed on a chlorophyll basis (c) in *Zea mays*. Each data point represents the mean of 4 to 6 replicates.

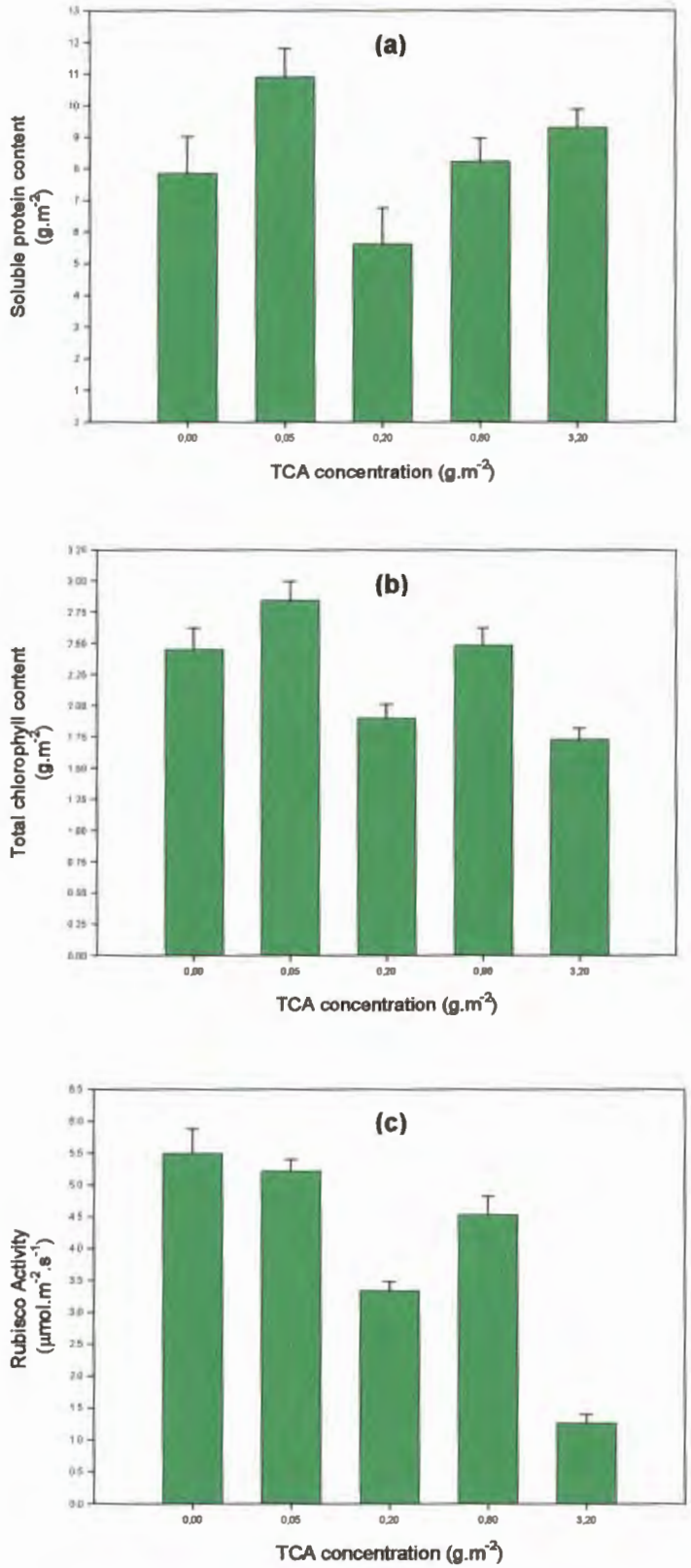


Figure 3.36 Effect of different TCA-concentrations on the total protein content per leaf area (a), total chlorophyll content per leaf area (b) and Rubisco-activity per sampled leaf disc (c) in *Zea mays*. Each data point represents the mean of 4 to 6 replicates.

As with high concentrations of TCA, TECE caused an increase in the maximum specific activity of Rubisco. However, TECE also caused an increase in initial specific activity, albeit to a lesser extent than in the case of the maximum activity. This resulted in a substantial decrease in the activation state of the enzyme (Figure 3.37b). Initial specific activity increased by 40% ($p<0.01$) and maximum specific activity by 57% ($p<0.01$) (Fig 3.37a). Similar to the observation at high TCA concentrations, the considerable increase in maximum activity could have been the result of the 51% decrease in the soluble protein content ($p<0.01$) in response to TECE treatment (Figure 3.38a). As was the case with the TCA-treatments, a true reflection of the response of Rubisco to TECE cannot be assessed accurately from activities expressed on a soluble protein basis. Since calculation of the activation state is dependent on these specific activities, the 26% ($p<0.05$) decrease observed after TECE-treatment, is not a reliable representation of the effect that it had on Rubisco (Figure 3.37b).

Expression of Rubisco activity on a total chlorophyll basis proved to be a more efficient way of evaluating the effect of TECE (Figure 3.37c), because the treatment did not induce such large changes in chlorophyll content (Figure 3.38b) as in the case of soluble protein content (Figure 3.38a). A decrease of 28% ($p<0.05$) occurred in the initial activity of Rubisco expressed on total chlorophyll content, thus illustrating that TECE induced an inhibition in Rubisco activity.

3.3.2.2.2. *Zea mays*:

The only significant changes observed were a 236% increase in the initial specific activity of Rubisco ($p<0.05$) (Figure 3.39a) and a 68% decrease in the soluble protein content ($p<0.05$) (Figure 3.40a). There was a 44% and 104% increase in the maximum specific activity of Rubisco and the activation state respectively, and decreases of 19%, 10% and 23% in the initial Rubisco activity expressed on a chlorophyll basis, total chlorophyll content, and initial Rubisco activity expressed on a leaf area basis, respectively. Even though some of these changes would seem to be very large, the large variation in values caused them not to be statistically significant.

3.3.2.3. Quantification of Rubisco protein content by Western Blot analysis after exposure to different TCA concentrations

Since the TCA treatments resulted in such significant effects on Rubisco activity and total soluble protein content, Western Blot analysis was conducted to determine the effect that TCA had on Rubisco protein content within the leaves of the two species. Following extraction of leaf protein, SDS-PAGE and protein transfer, Rubisco protein content was quantified using a primary antibody specific to the large subunit of Rubisco.

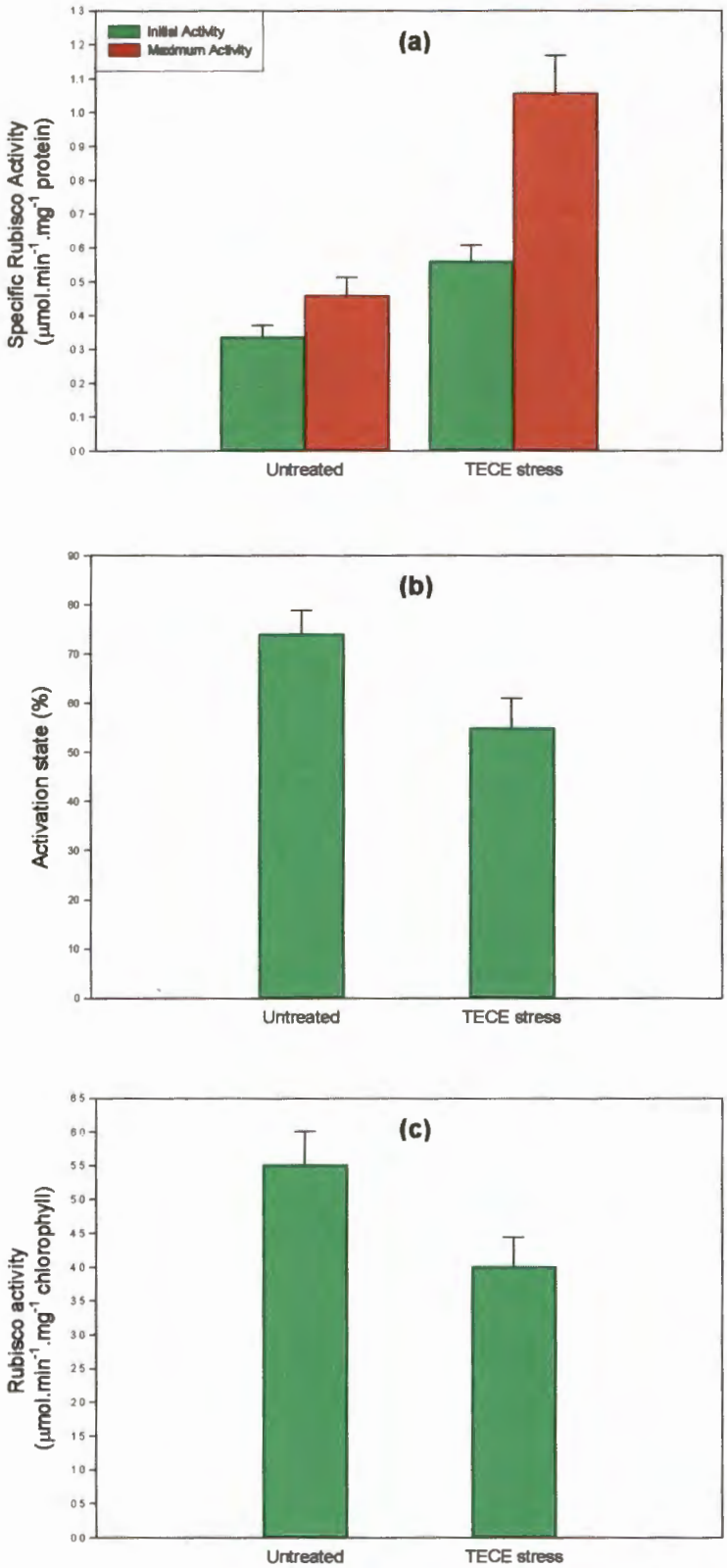


Figure 3.37 Effect of one week exposure to 810 µg TECE/m^3 on the initial and maximal activity rate of Rubisco (a), activation state (b) and Rubisco-activity expressed on a chlorophyll basis (c) in *Phaseolus vulgaris*. Each data point represents the mean of 4 to 6 replicates.

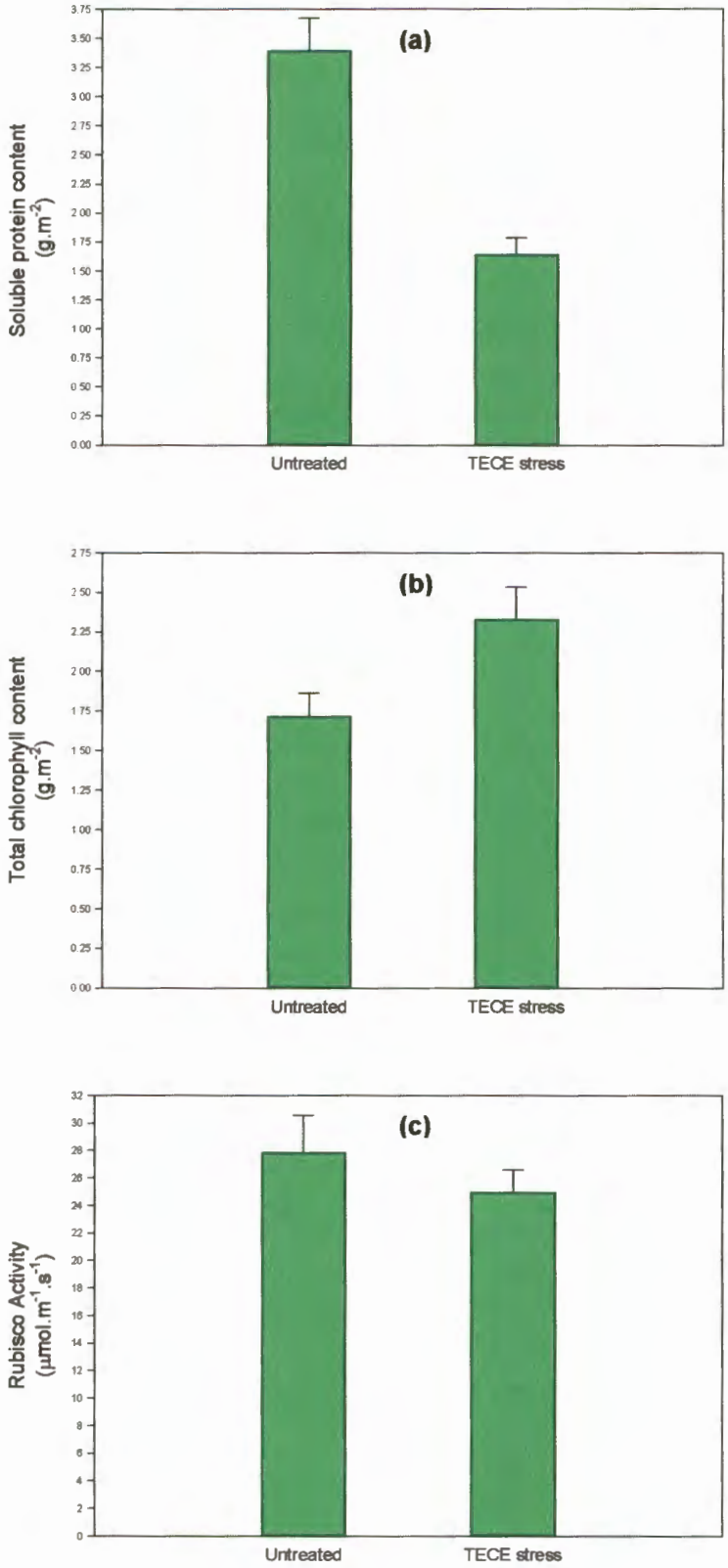


Figure 3.38 Effect of one week exposure to 810 µg TECE/m^3 on the total protein content per leaf area (a), total chlorophyll content per leaf area (b) and Rubisco-activity per sampled leaf disc (c) in *Phaseolus vulgaris*. Each data point represents the mean of 4 to 6 replicates.

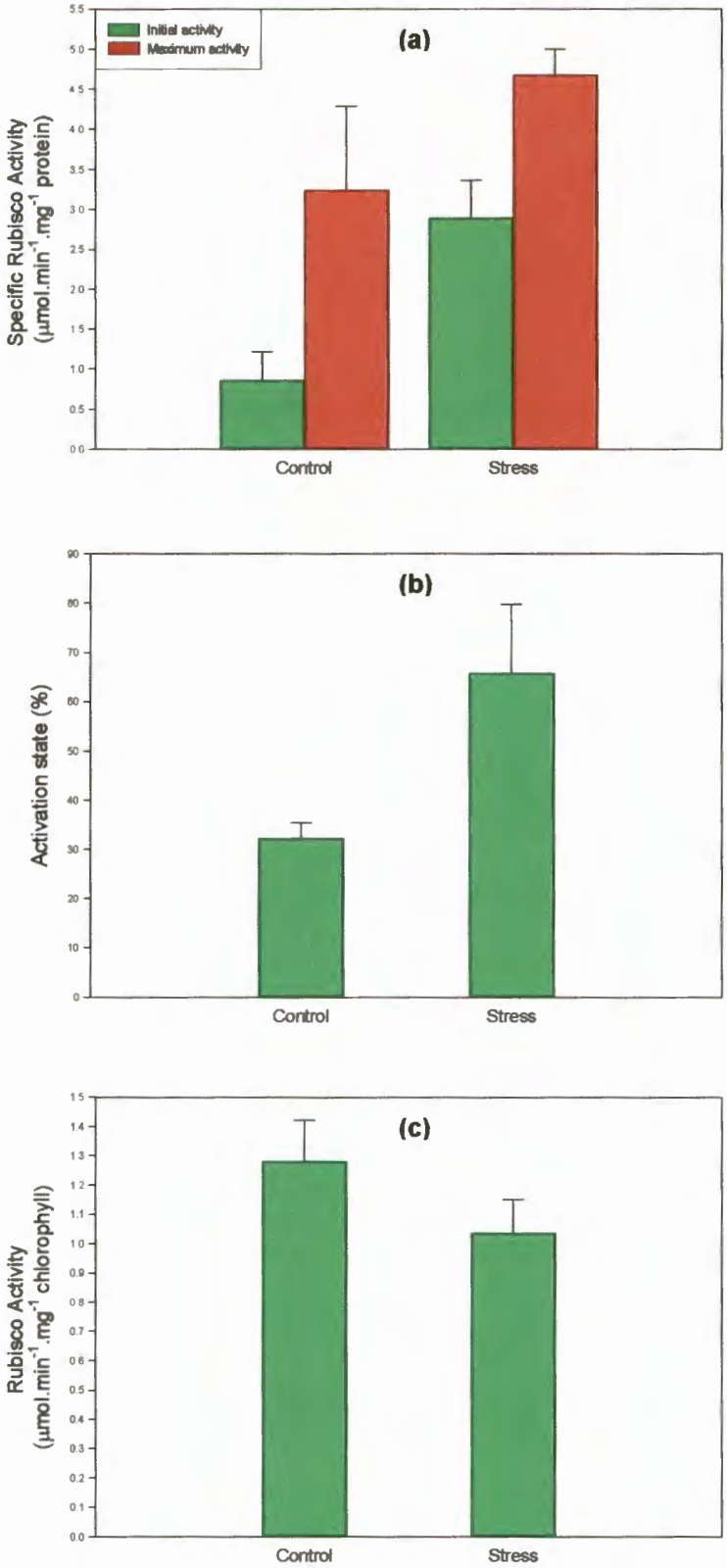


Figure 3.39 Effect of one week exposure to 810 µg TECE/m^3 on the initial and maximal activity rate of Rubisco (a), activation state (b) and Rubisco-activity expressed on a chlorophyll basis (c) in *Zea mays*. Each data point represents the mean of 4 to 6 replicates.

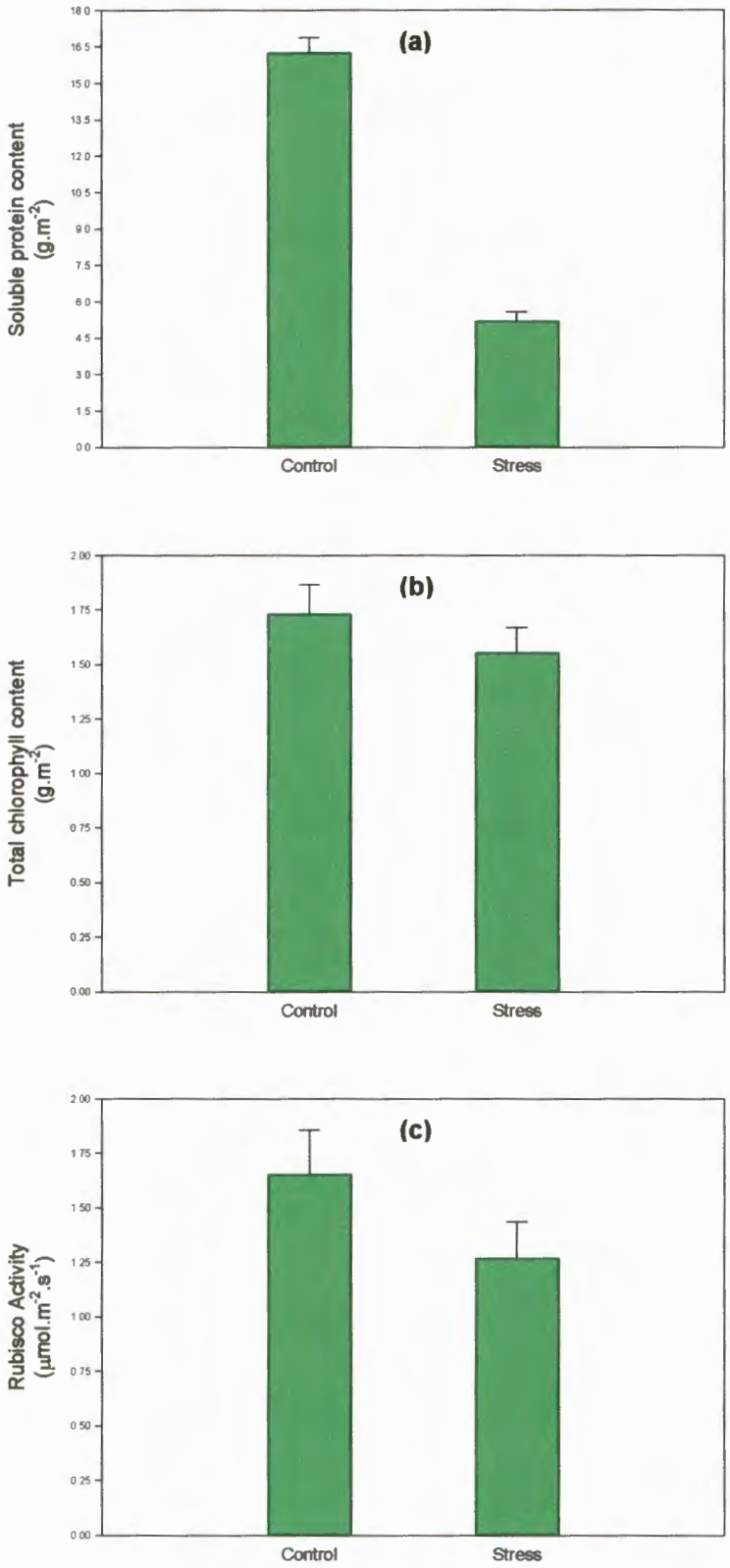


Figure 3.40 Effect of one week exposure to 810 µg TECE/m^3 on the total protein content per leaf area (a), total chlorophyll content per leaf area (b) and Rubisco-activity per sampled leaf disc (c) in *Zea mays*. Each data point represents the mean of 4 to 6 replicates.

3.3.2.3.1. *Phaseolus vulgaris*

The abundance of the large subunit of Rubisco in leaves from the control plants and plants treated with the different TCA concentration is shown in Figure 3.41. In each case a single band of approximately 45 kDa was revealed.

For each treatment the Rubisco protein content of the band on the Western Blot was estimated by means of image analysis. This was done by first determining the intensity of the band revealed by purified Rubisco of a known concentration (Rubisco standard). The intensity of the band at each TCA treatment was then compared to the intensity of the Rubisco standard. The Rubisco protein content for each treatment is shown in Figure 3.43a. Treatment with TCA resulted in an increase in Rubisco protein content of 13% at 0.05 g TCA/m^2, a smaller increase of 9% at 0.20 g TCA/m^2, a decrease of 11% at 0.80 g TCA/m^2, and no change at 3.20 g TCA/m^2. Taken together, these results suggests that there was no consistent effect on Rubisco protein content and that the changes that did occur, were of a much smaller magnitude than the effect of TCA on total soluble protein content. Thus although Rubisco protein content remained quite stable during treatment with TCA, its activity was decreased significantly, suggesting a decrease in the efficiency of Rubisco function.

3.3.2.3.2. *Zea mays*:

The abundance of the large subunit of Rubisco in leaves from the control plants and plants treated with the different TCA concentration is shown in Figure 3.42. In each case a single band of approximately 45 kDa was revealed.

In contrast to the small and inconsistent effects observed on *Phaseolus vulgaris*, Rubisco protein content of *Zea mays* showed a very large and clear pattern of change in response to TCA treatment. At the lower TCA concentrations, Rubisco protein content increased by 57% at 0.05 g TCA/m^2, by 152% at 0.20 g TCA/m^2 and by 145% at 0.80 g TCA/m^2. However, at the very high TCA concentration of 3.20 g TCA/m^2, the content decreased to a value 20% below that of the control (Figure 3.43b).

These results suggest an up-regulation of Rubisco expression and translation *in vivo*, possibly in an attempt to counteract the negative effects of TCA on Rubisco activity. This phenomenon seems to have been quite efficient, because Rubisco activity was affected to a very small extent at low TCA concentrations in *Zea mays*. However, from the results presented, it is clear that high TCA concentrations resulted in severe degradation of Rubisco protein with a concomitant decrease in Rubisco activity, especially when activity was expressed on a total chlorophyll or leaf area basis.

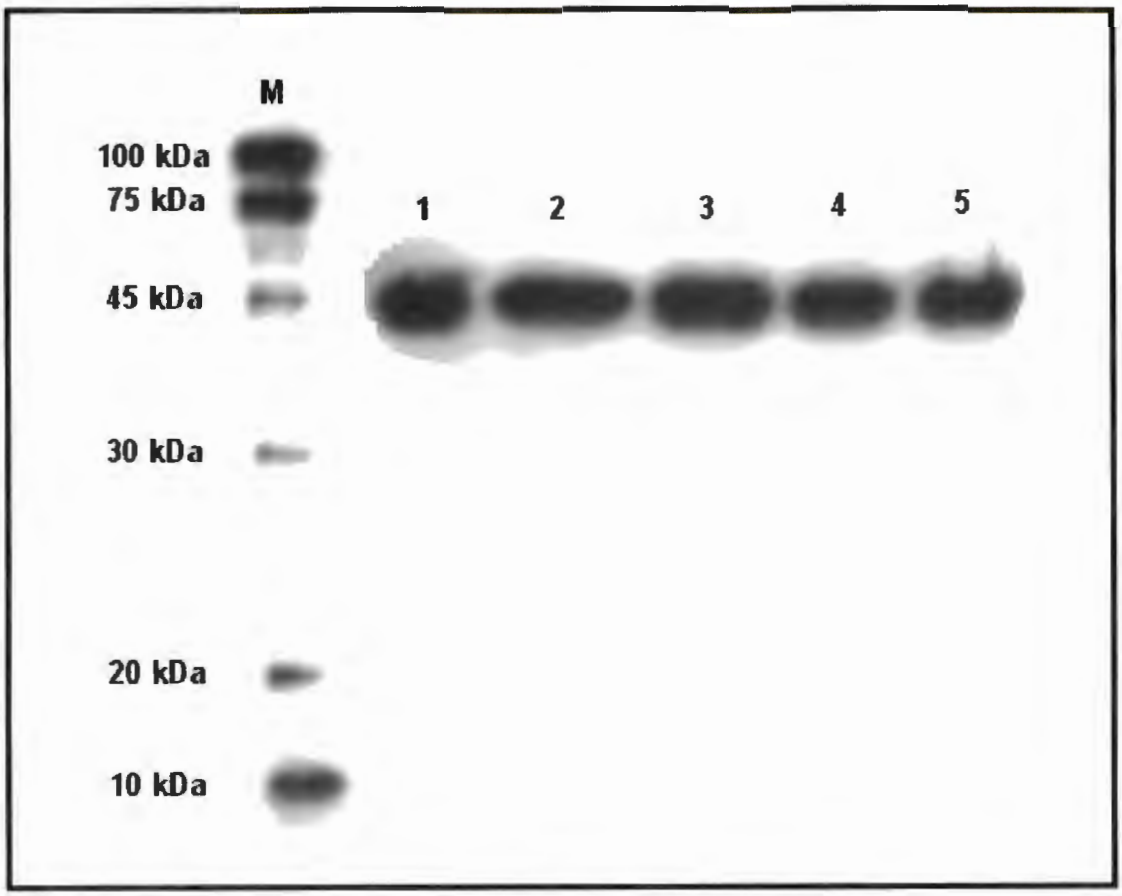


Figure 3.41 Western blot illustrating the expression of the large subunit of Rubisco in *Phaseolus vulgaris* exposed to different TCA concentrations. M = molecular weight markers; 1 = control/reference; 2 = 0,05 g TCA/m^2; 3 = 0,20 g TCA/m^2; 4 = 0,80 g TCA/m^2; and 5 = 3,20 g TCA/m^2.

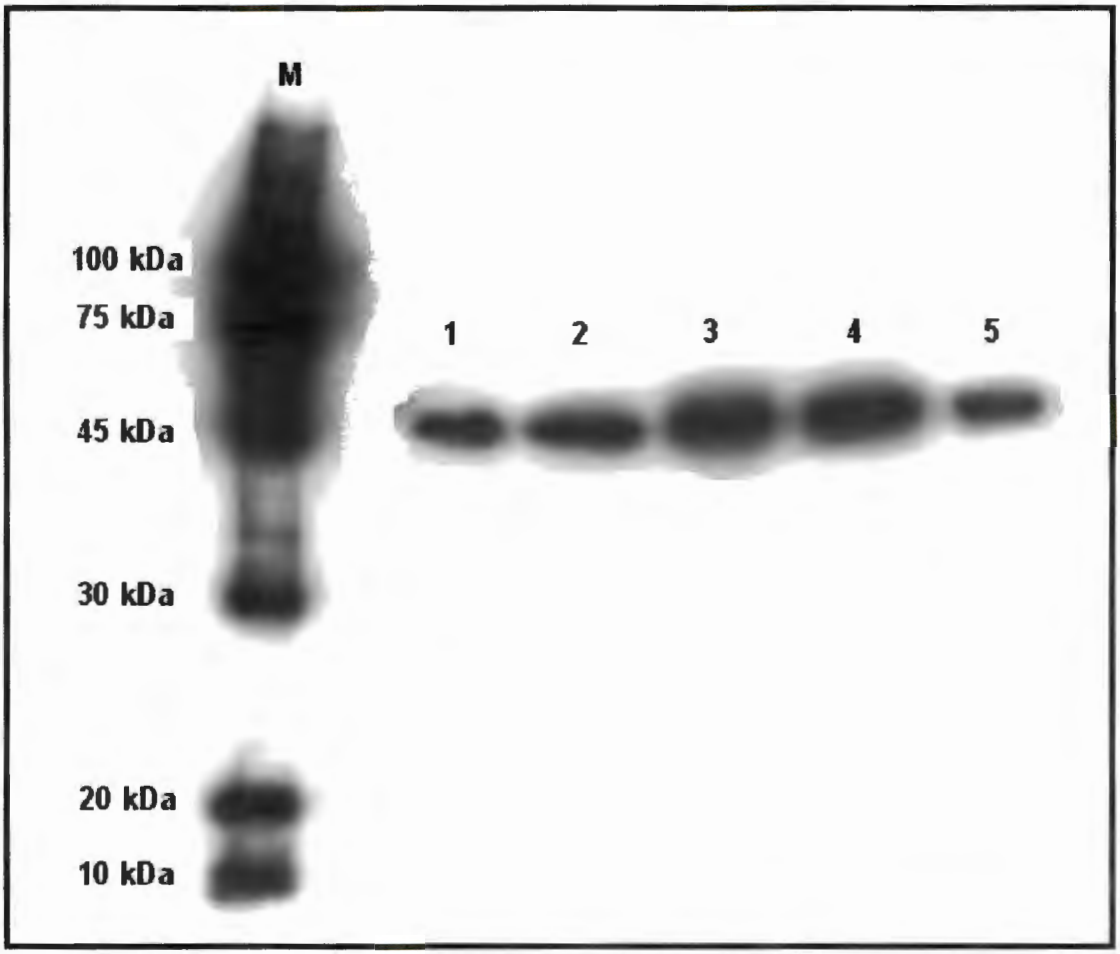
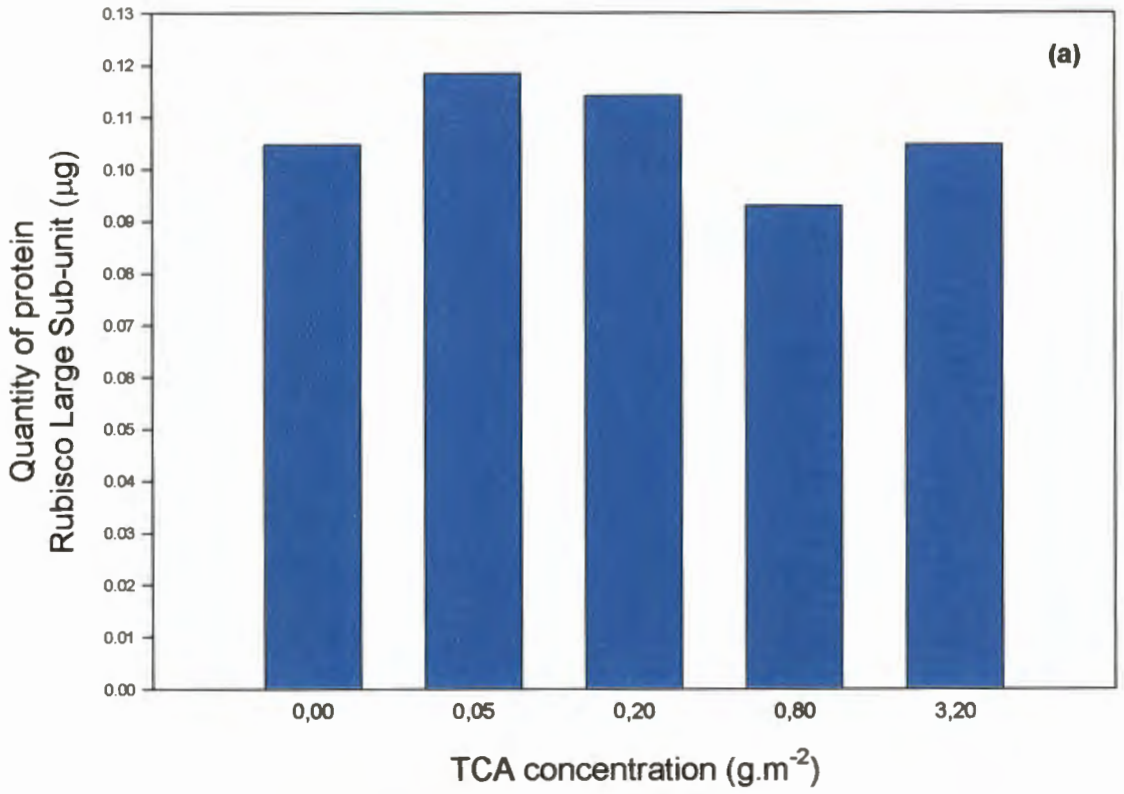


Figure 3.42 Western blot illustrating the expression of the large subunit of Rubisco in *Zea mays* exposed to different TCA concentrations. M = molecular weight markers; 1 = control/reference; 2 = 0,05 g TCA/m^2; 3 = 0,20 g TCA/m^2; 4 = 0,80 g TCA/m^2; and 5 = 3,20 g TCA/m^2.



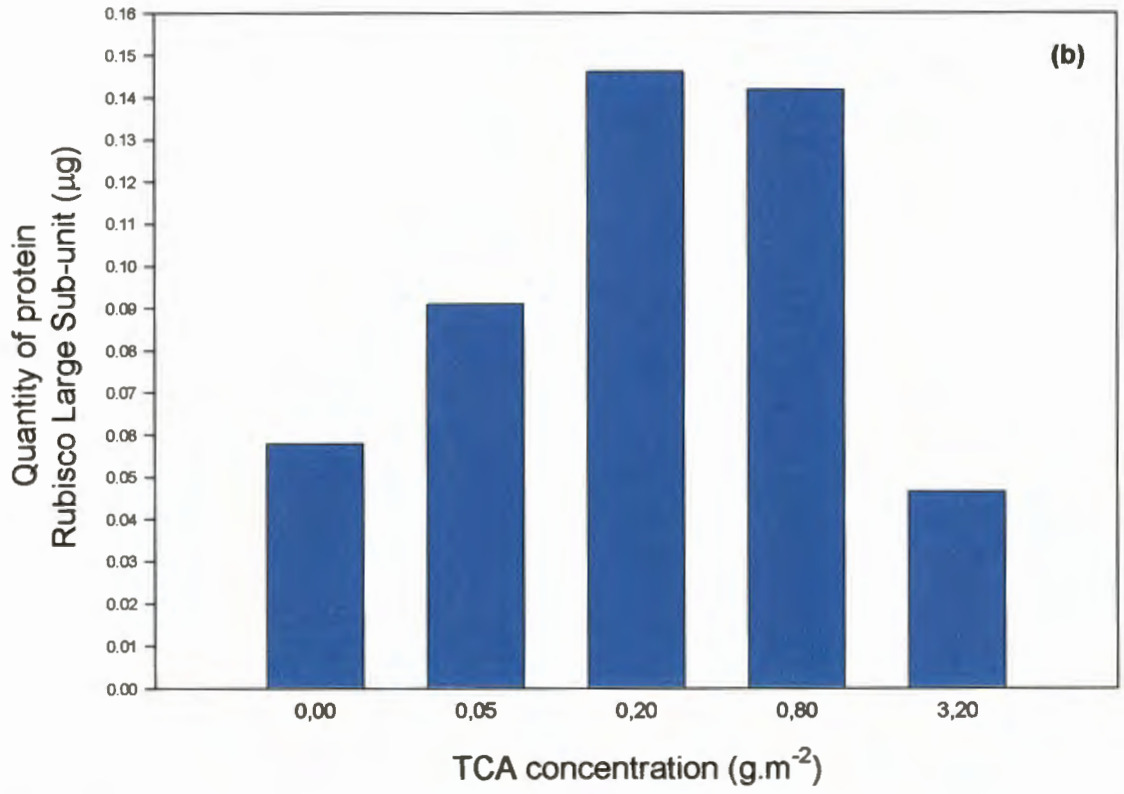


Figure 3.43 Quantity of the large subunit of Rubisco in (a) *Phaseolus vulgaris* and (b) *Zea mays* exposed to different TCA concentrations, expressed as protein content in µg, as determined with a Western blot analysis.

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Chapter 4:
Pine needles as bioindicator of the level of airborne TCA pollution and assessment of its phytotoxic effect:

4.1. Introduction:

4.1.1. Vegetation as monitor of air pollution:

According to Calamari *et al.* (1991) a linear relationship exists between the concentration of chemicals in foliage and in the air, and the concentration in foliage thus is a suitable measure of atmospheric contamination.

Many researchers have used pollutant concentrations in vegetation to qualitively indicate atmospheric contamination levels. Most of these attempts have been successful as vegetation integrates contamination over time. Furthermore vegetation samples are much easier to collect than air samples, especially in remote locations. Vegetation has been used to identify point sources of organic pollutants, to determine regional contamination within cities, countries, and continents, and to determine the global contamination of organic pollutants (Simonich and Hites, 1995).

However, the use of vegetation as bioindicators is more valid if the mechanism of plant uptake of organic pollutants is considered, thus keeping in mind the effect of temperature, vapour-particle partitioning, air concentration, plant species, and lipophilicity of the uptake of organic pollutants by plants. If possible, plant pollutant concentrations should be normalised to the plant lipid concentration or surface area, especially when comparing concentrations in different plant species (Simonich and Hites, 1995).

4.1.2. Using pine needles as bioindicators:

It seems that pine needles can provide a suitable sampling matrix for evaluating atmospheric contaminants (Eriksson *et al.*, 1989). This seems particularly true when determining regional exposures from a single-year and may also have relevance for trend analysis between years. The widespread distribution of the pine suggests that it may provide trend data for much of the world. The long life of the pine, compared to other biomonitors, potentially permits sampling from the same individuals over periods as long as one hundred years (Eriksson *et al.*, 1989).

Persistent air transported contaminants partition themselves between the vapour state and inert lipophilic materials. Pine needles provide such a matrix as they are covered with a wax coating which, on the whole, is similar for different locales and years and has been shown to contain a number of atmospherically transported pollutants. Furthermore, it is easy to identify the needles from three or more years and so collect data for several years in a single sampling. Contact with rainwater and absorption of the dissolved material by the needles is likely to be slight; dryfall particulate matter, although observed on needle surfaces, is believed to provide only a minor part of the contaminant burden of the needle wax. Translocation of the investigated compounds from the soil through the roots has been found to be a minor source of leaf residues in other plants but the significance of this route is not established for the pine. Diffusion within the wax layer is expected to be very slow because of the highly impermeable nature of the wax subsequent to emergence. Although background concentrations of the study compounds arising from global pollution are expected to be found in the needle wax, it should also show elevated levels when releases occur locally over a sufficient period to allow absorption. These conclusions are considered, although unproven in some instances, to be reasonable and conclude that contaminant concentrations in the needle wax will largely reflect time-integrated air concentrations. Indeed, meteorological effects aside, knowledge of these concentrations and the partition coefficients for wax/air, could provide a semi-quantitive estimate of the mean air concentrations (Eriksson *et al.*, 1989).

A two-compartment model that describes partitioning into the inner leaf separately best explains the kinetics of needle uptake. The two-compartment model includes a responsive surface compartment (the outer leaf) and a sluggish interior reservoir (the inner leaf). Initially, the majority of the pollutant is contained in the outer surface of the leaf, and then it gradually partitions into the inner compartment. Diffusion into the inner compartment is the rate-limiting step for whole leaf contamination (Simonich and Hites, 1995).

Taking into account the global concentrations of TECE specifically in the atmosphere, the partition coefficient of TECE between the compartments air/total lipid and wax of needles and the faster oxidation of TECE by OH radicals, indicates that atmospheric TECE concentrations up to about 500 km away from the emitter are especially important for the formation of TCA in plants (Weissflog *et al.*, 2001).

The diffusion of TCA, in particular, through epicuticular layers, plasmodesmata and cell membranes, is slow due to its total dissociation at neutral pH. The concentrations in the leaves and needles of different plant species from a polluted location listed in Table 4.1, argue against the majority of the TCA detected in the needles and leaves of various species being taken up via the soil/root pathway (Weissflog *et al.*, 2001). No significant difference existed between the TCA levels in the needles of Scots pine and Black pine. Since the leaves of *Betula pendula* have a higher stomatal and cuticular transpiration rate than the needles of

Scots pine and *Picea abies*, their greater sap flow ought to result in larger quantities of TCA reaching the plant via the soil/root pathway and accumulating in the leaf organs of the birch. This is, however, not the case, and thus provides further evidence countering the assumption that the soil/root pathway does not play the main role in the uptake of TCA at sites in the open countryside (Weissflog *et al.*, 1999).

Table 4.1 TCA levels (µg/kg fw) in the leaves and needles of trees (n=5) of various plant species at a polluted location (Golssen/Brandenburg, Germany) (Weissflog *et al.*, 2001).

Plant species	TCA concentration
Oak (*Quercus robur* L.)	3.4
Bird cherry (*Prunus avium* L.)	4.7
Birch (*Betula pendula* ROTH)	7.0
Acacia (*Robina pseudo-acacia* L.)	9.6
Red fire spruce (*Picea abies* L.)	23.5
Scots pine (*Pinus sylvestris* L.)	39.2
European black pine (*Pinus nigra* ARNOLD)	43.7

4.1.3. Accumulation of TCA in pine needles:

Simonich and Hites (1995) found that needles with high surface areas show higher rates of accumulation than needles with lower surface areas at equal exposure concentrations.

A case study by Frank *et al.* (1990) revealed that older pine needles exhibit higher TCA levels than younger ones, and there is a seasonal variation of TCA during the course of the year. The levels increase continuously until late autumn, and start to decline in early winter. TCA levels of several hundred ppb elicit extensive needle loss within a few months. The TCA present in plant tissue is likely to be of atmospheric origin, but oxidative formation of C_2-chlorohydrocarbons in the plant tissue is also possible.

TCA accumulates in pine needles with increasing needle age, and the concentration is reduced during winter. Concentrations of volatile chlorohydrocarbons in the needles mainly seem to reflect the actual emission loads. The seasonal development of TCA contents shows that either the atmospheric degradation or a supposed metabolisation of volatile chlorohydrocarbons inside the needles is reduced during the winter (Plümacher and Schröder, 1994).

4.1.3.1 A Case Study:

Simonich and Hites (1995) used Scots pine needles to trace emissions of TCA to two closely situated paper mills in Finland, which used ClO_2 as a bleaching agent. Samples were collected 5 to 120 km downwind from the mills and the TCA concentrations in needles were negatively correlated with the distance from the mills, thus indicating that the TCA concentrations in needles dropped dramatically within 10 km of the mills, but remained above background levels for 60-80 km downwind from the mills. The hypothesis is that TCA was produced by reactions of volatilised C_2-chlorohydrocarbons formed during the sewage treatment process, and using needles to indicate pollutant contamination levels, it was shown that pulp mills are sources of TCA when chlorine-containing bleaching agents are used.

4.2. Material and Methods:

4.2.1. Sampling:

Two year old pine needles were collected over two different transects, representing possible pollution gradients in Southern Africa in 2001. The vitality and TCA content of the sampled needles were subsequently determined.

Figure 4.1 Two year old needles collected from pine trees were used to determine their vitality and TCA content.

The first sampling transect started in the Northern Cape and continued northwest through Namibia, turning southeast through Botswana and ending in the Northwest Province. Sampling along these transects took place during March/April 2001 (Fig. 4.2).

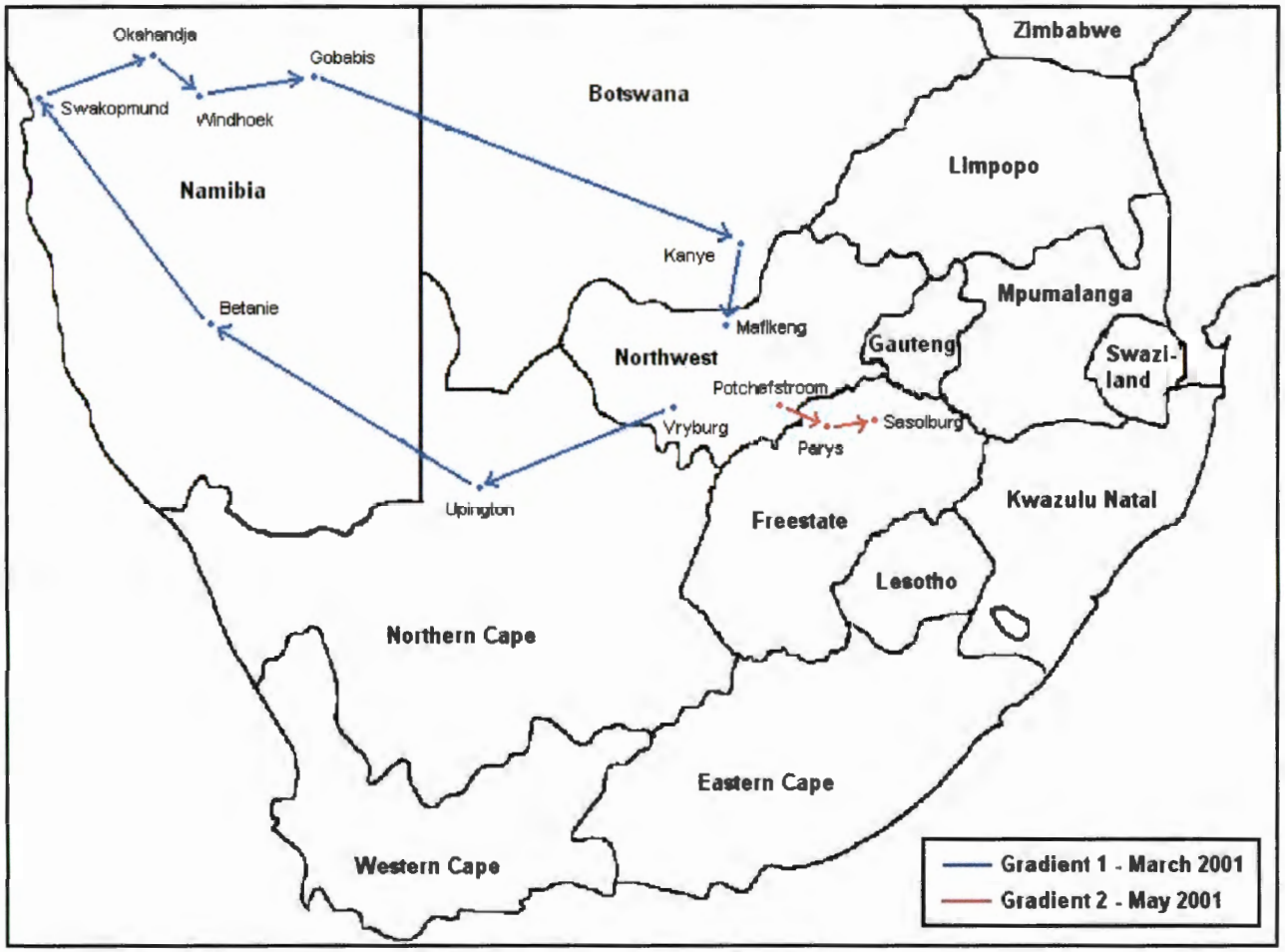

Figure 4.2 Pine needles were collected over two different gradients: the first starting in South Africa and stretching through Namibia and Botswana; and the second stretching from Potchefstroom through Parys to Sasolburg.

The second sampling transect extended over only 110 km, starting in Potchefstroom, extending southeast through Parys and ending in the highly industrialised part of Sasolburg. Sampling of the pine needles took place during May 2001 (Fig. 4.2).

4.2.2. Determination of TCA content of pine needles:

In plant tissue, organic acids may interfere with determination of TCA in the ppb-range. Analysis is therefore more appropriately performed by gas chromatography in conjunction with electron-capture detection or mass spectrometry.

Two-year-old needles were sampled, packed in airtight vials and stored at –20 °C until the TCA content was determined. A constant amount of 2.5 g needles was used for determination of the TCA in each sample. The analytical method used was based on the equilibrium of volatile compounds between the liquid or solid phase of the sample and the gas phase above it in a closed headspace vial. The equilibrium is dependent on the temperature, with higher temperatures causing higher concentrations in the gas phase.

An aliquot of the headspace gas was placed in a gas chromatograph. Since polar and water soluble compounds such as TCA cannot be measured directly by gas chromatography, the method of thermal decarboxylation of TCA to trichloromethane (chloroform, $CHCl_3$) was used. After the sample had been heated for 1 hour, the amount of $CHCl_3$ was measured (first injection). The second injection occurred after 72 hours when the TCA was completely decarboxylated. The TCA concentration was calculated as the difference between the $CHCl_3$ concentrations of these two injections (Weissflog *et al.*, 1999).

Measurements were carried out in the laboratory of Dr L Weissflog, Umwelt Forschungszentrum, (UFZ), Leipzig-Halle, Germany, by using a combination of a HP-7694 headspace sampler and HP-589111 gas chromatograph with an electron-capture detector. Headspace and GC conditions used are described by Weissflog *et al.*, 1999. The detection limit for trichloromethane is 0.2 µg/kg pine needles (fresh weight).

4.2.3. Chlorophyll Fluorescence measurements:

The same method and principles as discussed in 2.5 and 3.2.1 were used to determine the vitality of sampled pine needles, with a minimum number of 20 measurements taken for each tree at each sampling point.

4.3. Results and Discussion:

4.3.1. Pollution gradient 1 – Namibia transect:

Table 4.2 shows the TCA levels (µg/kg fresh weight) and corresponding vitality expressed by the PI_{ABS} and its components, of the needles collected at the different sampling sites.

No clear relationship existed between the TCA levels and the PI_{ABS} found in the needles of each sampling site (Fig. 4.3). One would have expected that low TCA content of needles is associated by relatively high PI_{ABS} values, and vice versa. This however, was true for some sampling points, but not all of them.

At the sampling points Upington, Betanie and Okahandja, only one composite sample was collected for TCA determination in each case, thus the representability of the sample may be questioned. Since a significant number of fluorescence measurements were taken at all the sampling points, the PI_{ABS} values obtained for each site can be regarded as a reliable reflection of the vitality of the needles at the particular site. Thus, if the PI_{ABS} measured at the sampling points where only one sample for TCA analysis was taken, corresponds with one of the PI_{ABS} values measured at a site where several samples for TCA analysis were taken, the TCA value obtained at this site could be regarded as an indication of what the TCA value would be at the site where only one sample was taken.

Even though more samples for TCA analysis were taken at the other sampling sites, there were still differences in the values for TCA obtained at sites where there was no difference in the PI_{ABS}. For example, fluorescence measurements made at the sampling sites of Vryburg and Windhoek revealed very similar PI_{ABS} values. However, there is a significant difference ($p<0.01$) between the TCA content of these sites.

Also, at the sampling points where the highest values for PI_{ABS} were obtained, the corresponding TCA levels found in the needles at the sites would have been expected to be the lowest of all the sampling sites, but this was not the case. The site Gobabis had the highest PI_{ABS} value of all the sampling sites, but the TCA content detected in the sampled needles was not the lowest of all the sampling sites.

Regardless of whether the differences in the vitality of the pine trees along the transect was influenced by TCA pollution level, there are clear differences in the vitality of the needles according to the measured PI_{ABS} values. PI_{ABS} can be regarded as a powerful vitality index due to the fact that it represents all partial processes of primary photochemistry.

Thus, a clear inverse relationship between the TCA content and vitality of the sampled pine needles could not be established with definite confidence. Other factors could also be responsible for the differences in vitality. The difference in pine species used as bioindicators could have an effect on the observed TCA content (Simonich and Hites, 1995) and possibly also on the measured vitality. Also, the influence of other environmental factors could have resulted in the observed differences, since the samples were taken at different localities, with environmental conditions ranging from moderate to very hot and dry.

Table 4.2 TCA content (µg/kg fw) and corresponding vitality (expressed by the performance index, PI_{ABS}, and its components) of pine needles collected over a transect stretching from the industrialised regions in South Africa north-west ward through Namibia. Sampling sites in Botswana were included as well.

	Pine species	TCA-content (µg/kg fw)	PI_{abs}	10RC/ABS	$\varphi_{po}/(1 - \varphi_{po})$	$\psi_o/(1 - \psi_o)$
Vryburg	*Pinus sylvestris* L.	0.30	17.12	3.66	3.90	1.20
Upington	*Pinus canariensis* C. Smith	10.83	17.45	3.66	4.01	1.19
Betanie	*Pinus sylvestris* L.	1.14	10.46	3.20	3.26	1.00
Swakopmund	*Pinus sylvestris* L.	6.04	23.87	4.62	4.25	1.21
Okahandja	*Pinus canariensis* C. Smith	4.06	25.72	4.36	4.36	1.35
Windhoek	*Pinus canariensis* C. Smith	4.28	17.17	3.74	3.85	1.19
Gobabis	*Pinus canariensis* C. Smith	5.04	35.02	4.63	4.22	1.80
Kanye	*Pinus sylvestris* L.	1.63	30.41	4.31	4.44	1.59
Mafikeng	*Pinus sylvestris* L.	0.22	19.41	4.13	3.82	1.23

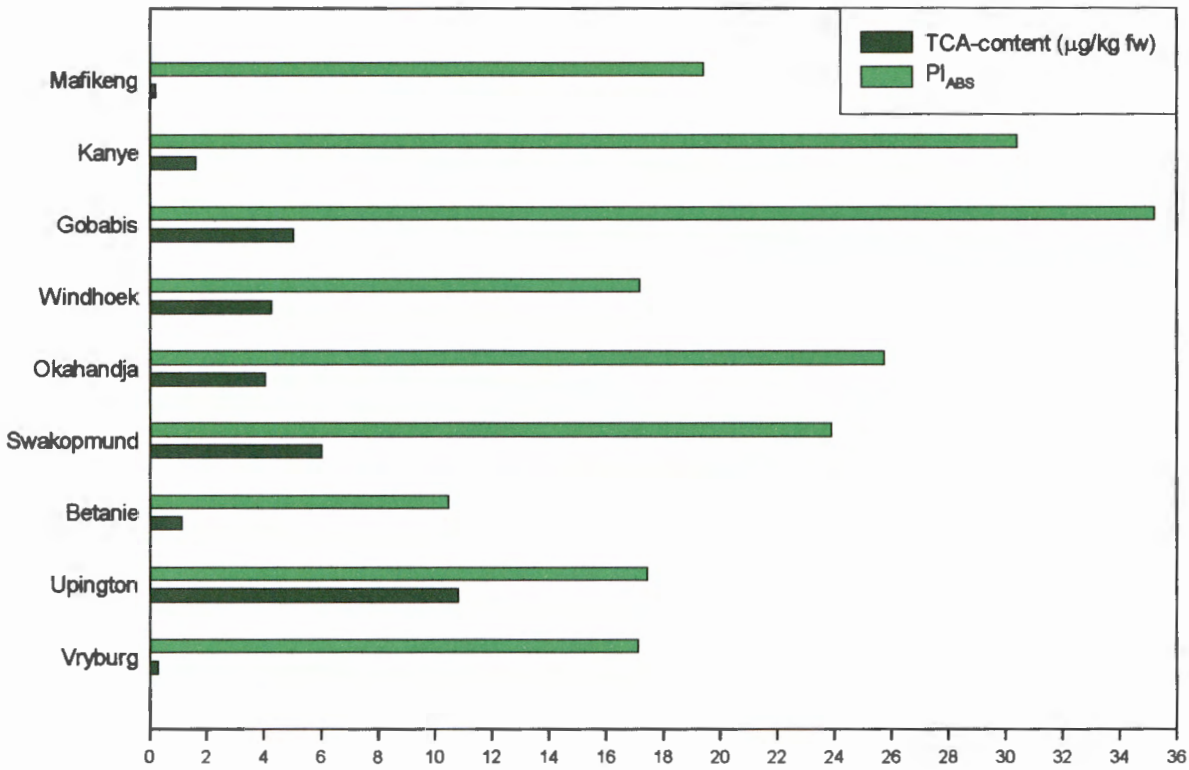

Figure 4.3 Content of TCA found in pine needles at each sampling site of the transect stretching from South Africa through Namibia and Botswana, with the corresponding performance index (PI_{ABS}) values obtained by the JIP-test.

4.3.2 Pollution gradient 2 – Potchefstroom – Sasolburg transect:

In Table 4.3 the levels of TCA found in the needles sampled over the gradient are given, together with the values of PI_{ABS} and its constituent components.

In the case of pollution gradient 2, a more definite pattern between the levels of TCA found in the sampled pine needles at each site and the measured values of PI_{ABS} (Figure 4.4) could be observed. Levels of TCA in the pine needles decreased with distance from the possible pollution source in Sasolburg, and vitality subsequently increased with distance from the source. In this case a clear inverse relationship existed between TCA content of the needles and their vitality expressed as PI_{ABS} (Figure 4.6).

The pine needles collected at the fourth sampling point in Sasolburg (Sasolburg #4) had the highest TCA content (10.24 µg/kg fw), rendering it as the possible source of the pollution. The data indeed show that the lowest vitality (PI_{ABS}) was measured at this specific sampling point (9.13). On the other extreme the highest PI_{ABS} value was measured at the Potchefstroom sampling site, namely 24.59, which coincided with one of the lowest TCA levels, namely 2.63 µg/kg fw. Similar TCA levels of 2.50 and 2.72 µg/kg fw were found at the second Sasolburg sampling site (Sasolburg #2) and the sampling site situated between Sasolburg and Heilbron respectively. The PI_{ABS} measured at these sites were not as high as that found at the Potchefstroom site, but the values were the second highest of the entire sampling transect, namely 20.34 and 20.19 respectively.

At the following four sampling sites very similar levels of TCA in the pine needles were obtained: between Potchefstroom and Parys (4.47 µg/kg fw), Parys (4.22 µg/kg fw), between Parys and Sasolburg (4.44 µg/kg fw), and Sasolburg sampling site #5 (4.64 µg/kg fw). These levels were higher than those found at Potchefstroom, Sasolburg #2 and Sasolburg/Heilbron. Thus theoretically, the PI_{ABS} measured at the four sites should be lower than the values found at the abovementioned sites. This was indeed the case, with PI_{ABS} values being 16.37 for Potchefstroom/Parys, 16.70 for Parys, 15.79 for Parys/Sasolburg, and 15.80 for Sasolburg #5 (Table 4.3, Figure 4.4).

For the remaining sampling sites a direct inverse relationship existed between TCA content and vitality (PI_{ABS}) of the needles. Figure 4.7 shows the % change in the TCA content and PI_{ABS} of the sampled pine needles relative to the average value obtained for each parameter. The inverse relationship existing between TCA content and PI_{ABS} is clearly illustrated here, and the decrease in TCA content and subsequent increase in vitality with increasing distance from the possible emitter source (Sasolburg #4) also comes to light.

Looking at the three constituent parameters of the PI_{ABS}, namely energy absorbance, trapping and electron transport, it would seem that the only parameter that followed the same pattern

Table 4.3 TCA content (µg/kg fw) of pine needles collected over a transect stretching from Potchefstroom through Parys to Sasolburg, with vitality according to the performance index and respective parameters.

	Pine species	TCA-content (µg/kg fw)	PI_{abs}	10RC/ABS	$\varphi_{po}/(1 - \varphi_{po})$	$\Psi_o/(1 - \Psi_o)$
Potchefstroom	*Pinus radiata* D. Don.	2.63	24.59	4.52	4.50	1.21
Potch-Parys	*Pinus sylvestris* L.	4.47	16.37	4.39	3.89	0.93
Parys	*Pinus canariensis* C. Smith	4.22	16.70	3.81	3.31	0.83
Parys-Sasol	*Pinus sylvestris* L.	4.44	15.79	3.92	4.01	1.00
Sasolburg #1	*Pinus sylvestris* L.	6.08	13.60	4.07	3.45	0.97
Sasolburg #2	*Pinus canariensis* C. Smith	2.50	20.34	4.33	3.84	0.90
Sasolburg #3	*Pinus sylvestris* L.	8.07	13.27	4.50	3.92	0.85
Sasolburg #4	*Pinus sylvestris* L.	10.24	9.13	4.00	3.34	1.01
Sasolburg #5	*Pinus sylvestris* L.	4.64	15.80	4.16	3.58	1.06
Sasol-Heilbron	*Pinus canariensis* C. Smith	2.72	20.19	4.55	4.29	1.03

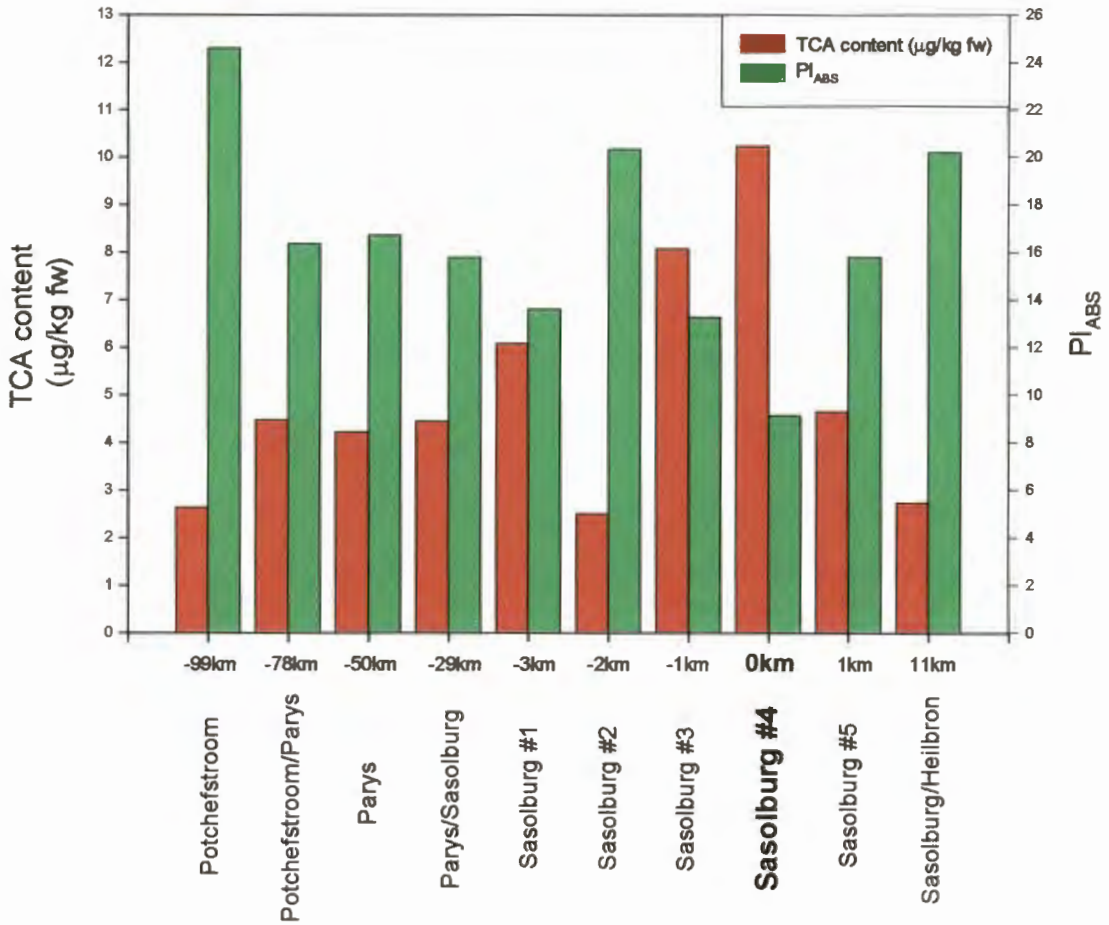

Figure 4.4 TCA content (µg/kg fw) and vitality (expressed as PI_{ABS}) of sampled pine needles, with increasing distance from the possible source of pollution in Sasolburg.

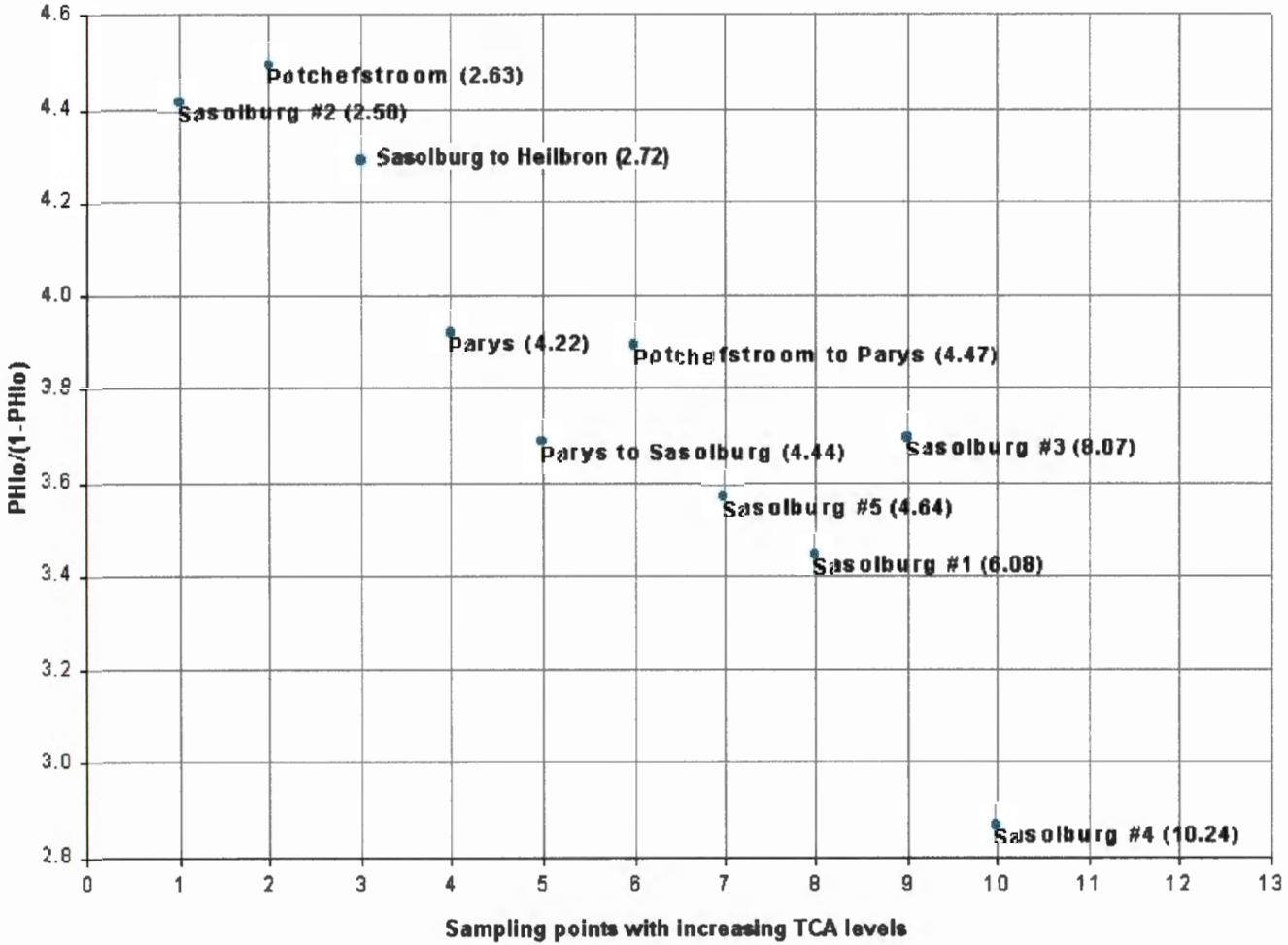

Figure 4.5 Dependence of efficiency of excitation energy trapping (PHI_o/1-PHI_o) on TCA content (µg/kg fw) of pine needles. The location where the needles were sampled is indicated.

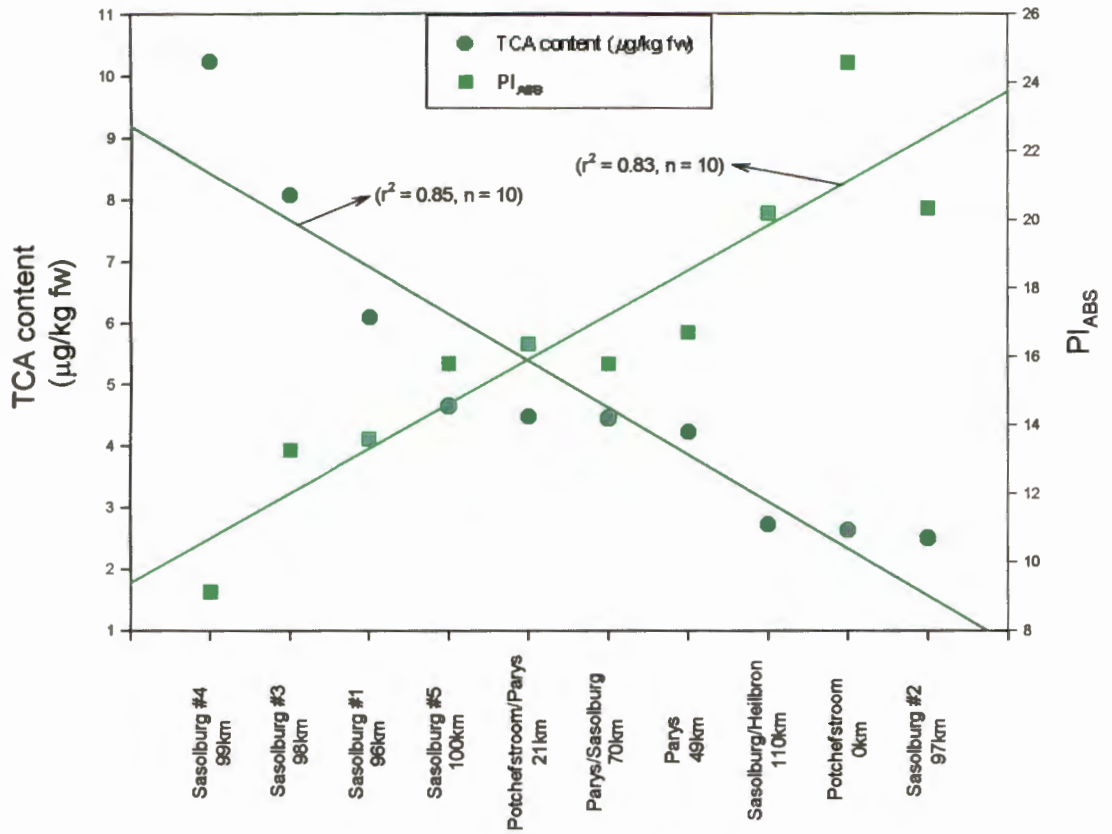

Figure 4.6 Illustration of the inverse relationship existing between the decrease in TCA content of pine needles (µg/kg fw) and the increase in vitality, expressed as PI_{ABS}.

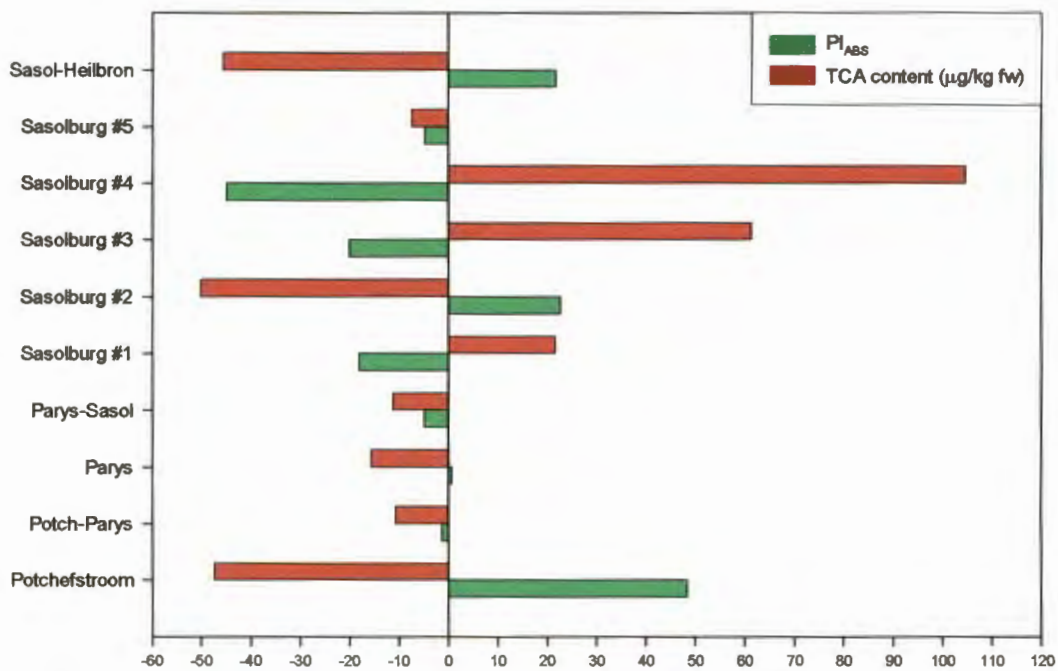

Figure 4.7 PI_{ABS} and TCA content (µg/kg fw) of pine needles collected over a pollution transect of 110km between Potchefstroom and Sasolburg, relative to the average.

of change as the PI_{ABS}, relative to the increase in TCA content, was the partial driving force of trapping of excitation energy ($PHI_o/(1-PHI_o)$) (Fig. 4.5). Thus, the change exhibited by PI_{ABS}, presumably relative to the change in TCA levels in the pine needles, could be the result of an effect on the efficiency to trap excitation energy.

Although three different pine species were used as bioindicators, it did not seem to have an effect on the measured values of TCA and PI_{ABS}. At the Parys sampling point *Pinus canariensis* was used as bioindicator, and it exhibited similar PI_{ABS} and TCA values as the *Pinus sylvestris* samples taken at the Potchefstroom/Parys, Parys/Sasolburg and Sasolburg #5 sampling sites.

Since the samples were collected over a distance of about 110 km and in a time period of only one day, other than was the case with the Namibia transect, the environmental conditions of the different sampling sites were more comparable. Thus the influence of other environmental factors on the vitality of the pine needles was probably fairly similar, rendering the PI_{ABS} values obtained at the different measuring sites more comparable than in the case of the Namibia transect. In this case the difference in PI_{ABS} values of the different measuring sites could confidently be assumed to be the result of the phytotoxic effect of TCA on the plants.

In addition, in the above mentioned case more than one sample for TCA analysis were collected at each sampling site, rendering the average value for the TCA content of the pine needles at each site more reliable and representing.

4.4. Conclusion:

To succesfully use pine needles as bioindicators for e.g. TCA pollution, it is important to consider all other factors that could possibly have an effect on the vitality of the plants. Thus the immediate environment of the trees from which samples are collected must be taken into account when interpreting the data. For more confident statistical verification of differences between the TCA content of pine needles from different sampling sites, enough samples for TCA analysis should be collected at each sampling site.

Although the Namibia transect did not reflect a definite relationship between the levels of TCA in the pine needles and the vitality of the plants, it was clear that the vitality of the vegetation from the different sampling sites differed due to a factor, which could have been a summation of the effect of TCA and other environmental effects. If more samples for TCA analysis had been collected at all the sites, a more reliable relationship between the TCA levels and the vitality of the plants could have been established.

Data obtained along the transect measuring eastward from Potchefstroom, through Sasolburg, in the direction of Heilbron, clearly reflected the inverse relationship between TCA content of the pine needles and their vitality expressed by the PI_{ABS} value. Furthermore, the data demonstrated that a pollution gradient with regard to TCA pollution exists along this transect, with the possible source of pollution being situated at the fourth sampling site in Sasolburg. Consideration of all the factors contributing to the vitality of the plants revealed a possible inhibition of trapping of excitation energy. Thus it might be concluded that deposition of TCA on, and the subsequent uptake thereof by natural vegetation, could have a major effect on the plants' photosynthesis, and more specifically the primary processes of photochemistry.

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Chapter 5: Conclusion:

Exposure of plants to the C_2-chlorohydrocarbon tetrachloroethene and its metabolite trichloroacetic acid, to which it is atmogenically transformed under natural conditions, results in inhibition of the photosynthetic metabolism. This is true for crop plants as well as natural vegetation. Primary photochemistry, the dark reactions of photosynthesis, as well as photosynthetic CO_2 gas exchange are affected to a greater or lesser degree.

The following conclusions could be made after treatment of the C_3 and C_4 crop plants, *Phaseolus vulgaris* and *Zea mays* respectively, with TCA under controlled conditions:

- Low TCA concentrations (<0,50 g TCA/m^2) had a stimulatory effect on the growth and metabolism of *P. vulgaris*. Both the rate of CO_2 assimilation and carboxylation efficiency increased at lower TCA-dosages, with inhibition commencing at increasing concentrations. Stomatal limitation however increased from the lowest concentration with increasing TCA-dosage. Similarly, the protein- and chlorophyll content also showed an initial stimulation, followed by a decrease at higher TCA-levels. This phenomenon of initial stimulation at low TCA concentrations was corroborated by the chlorophyllI fluorescence data. TCA would seem to affect photosynthesis in a C_3-plant such as *P. vulgaris* by inhibition of mesophyll processes, i.e. primary photochemistry and/or enzymatic reactions, at high concentrations.
- The metabolism of *Z. mays* was inhibited, even at the lowest TCA concentrations used. Marked decreases in photosynthetic CO_2 gas exchange, specifically the rate of assimilation (carboxylation efficiency) occurred, pointing at mesophyll limitation. Coincidingly, Rubisco activity remained stable at lower TCA-dosages, but higher levels caused degradation of the protein. The vitality of *Z. mays*, expressed as the vitality index (PI_{ABS}), showed a similar response to TCA as in the case of *P. vulgaris*, namely a very slight stimulation at low concentrations and inhibition at increasing levels. The process of primary photochemistry affected most upon exposure to TCA in the C_4-plants (*Z. mays*) would seem to be the efficiency of converting excitation energy to electron transport beyond Q_A.

It could be concluded that although one week of exposure to TECE vapour under controlled conditions affected the treated plants to a lesser extent than its metabolite, TCA, certain metabolic parameters showed marked effects:

- TECE-induced inhibition of mesophyll processes was evident in *P. vulgaris*. A large decrease in carboxylation efficiency and concomitant increase in the levels of intercellular CO_2 concentration at ambient CO_2, as determined *in vivo* by $A:C_i$ response curves, pointed at reduced Rubisco activity, as was proved by *in vitro*

enzyme analysis, to be indeed the case. The overall vitality of *P. vulgaris* was not affected markedly by TECE treatment, although a decrease in the density of reaction centres, as well as a reduction in the conversion of trapped excitation energy to electron transport, was observed.

- The C_4 plant (*Z. mays*) seemed to be more resistant to TECE treatment than *P. vulgaris*. No clear changes in photosynthetic CO_2 gas exchange occurred, and the activity of Rubisco was affected to a lesser extent. This phenomenon should be ascribed to the specialised anatomy of the leaves of *Z. mays*, which uses PEP carboxylase as the initial enzyme responsible for CO_2 fixation, and the fact that Rubisco is shielded from the outside atmosphere due to its location in the tightly packed bundle sheath cells. Nevertheless, the primary photochemistry showed clear indications of inhibition, with decreases in most of the key processes of photosynthesis contributing to the vitality index (PI_{ABS}). The efficiency with which excitation energy is trapped and subsequently converted to electron transport proved to be most affected.

By assessing the effect of TCA on natural vegetation by utilising two year old pine needles from sampling sites as bioindicator for the levels of TCA pollution over a presumed pollution gradient, a direct correlation between plant vitality and TCA content in the needles could be made, leading to the following conclusions:

- No clear relationship between plant vitality and TCA content could be established for the transect from South Africa to Namibia. This must be ascribed to the wide diversity of environmental conditions at the different sampling sites. Nevertheless, the vitality of the plants at the different sites on the transect was affected by an unknown factor, which could be the effect of TCA in combination with another stress.
- The transect studied from Potchefstroom to Sasolburg showed more definite evidence of a possible TCA pollution gradient, since an inverse relationship existed between TCA content of the sampled pine needles and their vitality. The levels of TCA in the pine needles decreased with increasing distance from the possible emitter source in Sasolburg, and concomitantly their vitality increased.
- On a photosynthetic level it would seem that the efficiency to trap excitation energy is the process of primary photochemistry affected most by exposure of natural vegetation to TCA pollution.

The data of this investigation convincingly indicated that the non invasive and rapid JIP-test can successfully be applied to assess:

- The effect of airborne C_2-chlorohydrocarbon pollution on both natural vegetation and crop plants.
- The physiological and biochemical basis of the phytotoxic effect of pollutants on plants. A linear and indirect relationship existed between the TCA concentration

applied to the test plants and their vitality expressed by the chlorophyll fluorescence deduced performance index (PI_{ABS}).

The data obtained by concurrent determination of the TCA content and vitality of pine needles over a known air pollution gradient, reconfirmed the successful application of pine needles, providing that other environmental variables are also taken into account, to assess the levels of airborne C_2-chlorohydrocarbons.

Thus it could be concluded that C_2-chlorohydrocarbon pollution (such as TECE) has a direct inhibitory effect on vegetation. Furthermore, the subsequent transformation to, and deposition of phytotoxic compounds like TCA on vegetation, has very definite effects on the photosynthetic metabolism of both crop plants and natural vegetation. Although it would seem that C_4 plants are better equipped to deal with the stress of pollution by C_2-chlorohydrocarbons like TECE, exposure to and uptake of TCA through the root system seems to inhibit C_4-plants, even at very low concentrations. However, when a C_3 plant (such as *P. vulgaris*) is exposed to very low concentrations of pollution by TCA in particular, they exhibit a degree of stimulation in both growth and metabolism, but this initial stimulation was nullified at higher concentrations. Nevertheless, when the effects that these pollutants have on vegetation act in combination with additional environmental stress factors like drought, it could lead to severe damage and possible desertification in sensitive ecosystems like grasslands.

Appendix A:

Conference contributions that resulted from this project:

Seminar/Workshop on Urban and Settlement Ecology

Potchefstroom, 24 & 25 August 2001

A.J. Strauss & G.H.J. Krüger

Quantification of the response of vegetation to environmental stresses.

South African Academy for Science and Art: Division Biological Sciences: Annual Congress

Potchefstroom, 4 October 2001

A.J. Strauss & G.H.J. Krüger

Die invloed van trichloorasynsuur op die fotochemie en fotosintetiese CO_2-fiksering van C_3- en C_4-landbougewasse.

Alternative Ways to Combat Desertification: Connecting Community Action with Science and Common Sense: International Congress

NBI, Kirstenbosch, Cape Town, 8-20 April 2002

A.J. Strauss, G.H.J. Krüger & L. Weissflog

TCA content and vitality of vegetation along air pollution gradients in Southern Africa: Implication for desertification and crop production.

South African Association of Botanists: Annual Congress

Pretoria, 8-11 January 2003

A.J. Strauss, P.D.R. van Heerden & G.H.J. Krüger

The effect of the atmogenically produced phytotoxic C_2-chlorohydrocarbon metabolyte, trichloroacetic acid, on photosynthesis of crop plants.